Capsaicin and Vanilloid Receptors in the Perfused Rat Hindlimb: Mechanisms of Action

by

David
Cory D. Griffiths BSc (Hons)

Submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy

Division of Biochemistry / School of Medicine
University of Tasmania (October, 1998)

DECLARARTION

This thesis contains material which has not been accepted for the award of any other degree or diploma, except where due acknowledgment is given. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made.

C.D. Griffiths

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PREFACE AND ACKNOWLEDGMENTS

The majority of the data presented in this thesis was obtained between January 1995 and March 1998 during my employment as a Graduate Research Assistant and Phd candidature (part-time) in the Division of Biochemistry, University of Tasmania. Some preliminary data for the effects of capsazepine, ruthenium red and tetrodotoxin on capsaicin-mediated responses in the perfused rat hindlimb were obtained during 1994. However, additional work leading to the publication of that data was performed within the time frame mentioned above.

It has been my pleasure and privilege to have been under the supervision of Associate Professor Eric Colquhoun throughout my PhD studies. His role as a mentor and friend have been invaluable in the completion of my studies and development of my research capability. I thank him in particular for maintaining his interest and enthusiasm in my project despite the heavy demands of running a full-time general practice and family.

I am equally grateful to my associate supervisor and good friend Dr. Dominic Geraghty, Department of Biomedical Science, University of Tasmania. His broad knowledge of the tachykinins and pharmacology, together with his likeable Irish personality, have been an essential components of my professional and personal development. I thank him a great deal for the many light-hearted moments that have helped keep me sane over the years.

I would also like to thank Professor Michael Clark (Head of the Division of Biochemistry) who has provided great support to me in addition to the supervision of his own PhD students, and heavy research and teaching load. I thank him in particular for allowing me to contribute to weekly research meetings, and for maintaining his interest in the vanilloids project despite its diversion from the mainstream studies conducted in his laboratory.

I also extend my gratitude to Dr. Tristram Eldershaw for his friendship and collaborative involvement in studies presented in Chapters 2 and 3, to Ms. Jenny Hall for her support and contribution to the tetrodotoxin studies in Chapter 2, and to Mr. Stuart Mazzone for his highly valued friendship and contribution to the autoradiographic studies presented in Appendix 1. I also thank Dr. Jiming Ye for his many helpful suggestions and advice.

I would like to express special thanks to Mr. John Jordan who's recent tragic death was a great shock and loss to all who knew him. His vast technical skills were essential for the maintenance and construction of laboratory equipment and he will long be remembered for his dedication and generous nature. Equally tragic was the sudden death this year of Dr. Alex Tong, with whom I experienced many of the highs and lows of both undergraduate and postgraduate studies. I have long admired the outstanding commitment Alex demonstrated in completing his PhD studies, and to his loving wife and children. I extend my deepest sympathies and best wishes to both families.

Other contributors to an excellent research environment have included Dr. Stephen Rattigan, Dr. Jenny Penschow, Dr. John Steen, Mr. Geoff Appleby, Ms. Kelly Miller, Ms. Michelle Vincent, Mr. John Newman, Ms. Joanne Youd, Ms. Marie Smith, Ms. Carla DiMaria, Ms. Lucy Clerk and Ms. Michelle Wallis.

I would like to acknowledge the generous gift of phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) donated by Drs. G. Appendino (Department of Pharmacology, University of Torino, Torino, Italy) and A. Szallasi (Department of Anatomy and Neurobiology, Washington University, St. Louis, USA); CP-99.994 donated by Dr. S.B. Kadin (Pfizer Inc., USA); and SR48968 donated by Dr. X. Emonds-Alt (Sanofi Recherche, France). I also thank Dr. Szallasi for his helpful correspondence by electronic mail.

Finally, I would like to thank my family, particularly my loving wife Justine who's help and support throughout my PhD candidature has been greatly appreciated.

The bulk of the data presented in this thesis have been published and/or presented at scientific meetings.

Publications arising from data presented in this thesis:

1. **GRIFFITHS, C.D.**, ELDERSHAW, T.P.D., GERAGHTY, D.P., HALL, J.L. AND COLQUHOUN, E.Q. (1996) Capsaicin-induced biphasic oxygen uptake in rat muscle: antagonism by capsazepine and ruthenium red provides further evidence for peripheral vanilloid receptor subtypes (VN_1/VN_2). *Life Sci.* **59**: 105-117.

2. **GRIFFITHS, C.D.**, GERAGHTY, D.P., ELDERSHAW, T.P.D., AND COLQUHOUN, E.Q. (1998) Acute and chronic effects of capsaicin in muscle: the role of tachykinins and calcitonin gene-related peptide. *J Pharmacol. Exp. Ther.* In press.

Related papers presented at scientific meetings:

C.D. GRIFFITHS, T.P.D. ELDERSHAW, D.P. GERAGHTY AND E.Q. COLQUHOUN Thermogenic and Thermoinhibitory Vanilloid (Capsaicin-like) Receptors in Rat Muscle. *Third Joint Scientific Meeting of the Australasian Society for the Study of Obesity and Australian Clinical Nutrition Society*, Melbourne, September 1995.

C.D. GRIFFITHS, T.P.D. ELDERSHAW, D.P. GERAGHTY AND E.Q. COLQUHOUN. Capsaicin-induced Oxygen Uptake in Rat Muscle via Substance P and NK1 Receptors. *30th Annual Scientific Meeting of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists,* Melbourne, December 1996.

C.D. GRIFFITHS, D.P. GERAGHTY, T.P.D. ELDERSHAW & E.Q. COLQUHOUN. Vanilloid Receptor Subtypes in Muscle: Different Mechanisms and Capsaicin Sensitivity. *International Tachykinin Conference: Tachykinins in Health and Disease,* Cairns, September, 1997.

C.D. GRIFFITHS, D.P. GERAGHTY, T.P.D. ELDERSHAW & E.Q. COLQUHOUN. Capsaicin pretreatment differentially regulates the responses of vanilloid receptor subtypes (VN_1/VN_2) in rat muscle. *31st Annual Scientific Meeting of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists,* Canberra, December 1997.

Cory D. Griffiths

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ABBREVIATIONS

α_1-AGP	alpha$_1$ acid glycoprotein (orosomucoid)
ACh	acetylcholine
AII	angiotensin II
ANOVA	analysis of variance
[^{125}I]-BHSP	[^{125}I]-Bolton-Hunter substance P
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCh	carbamyl choline (carbachol)
cDNA	copy DNA
CGRP	calcitonin gene-related peptide
$CGRP_{(8-37)}$	calcitonin gene-related peptide antagonist
Ci	Curie
CNS	central nervous system
CP-99,994	non-peptide NK1 receptor antagonist
CPZ	capsazepine
CSPAN	capsaicin-sensitive primary afferent neuron
CTX	ω-conotoxin GVIA
DRG	dorsal root ganglia
EC_{50}	half-maximal effective concentration
EDHF	endothelium-derived hyperpolarising factor
EDRF	endothelium-derived relaxing factor
E_{max}	maximal effective concentration
5-HT	5-hydroxytryptamine (serotonin)
IC_{50}	half-maximal inhibitory concentration
IMGU	insulin-mediated glucose uptake
i.p.	intraperitoneal
IU	international units
i.v.	intravenous
K_D	dissociation constant
L-NAME	Nω-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine

mRNA	messenger RNA
NK1/NK2/NK3	neurokinin (tachykinin) receptor subtypes
NKA	neurokinin A
NKB	neurokinin B
NO	nitric oxide
NOR	noradrenalin
NOS	nitric oxide synthase
NP	nitroprusside
P	probability
pA_2	log concentration of antagonist required to produce a two-fold shift to the right in a concentration-response curve
PO_2	oxygen partial pressure
PP	perfusion pressure
PPAHV	phorbol 12-phenylacetate 13-acetate 20-homovanillate
RP 67580	non-peptide NK1 receptor antagonist
RR	ruthenium red
RTX	resiniferatoxin
[^{3}H]-RTX	tritium radiolabelled resiniferatoxin
s.e.	standard error
S-N-K	Student-Newman-Keuls multiple comparison
SNS	sympathetic nervous system
SP	substance P
SR 48968	non-peptide NK2 receptor antagonist
TTX	tetrodotoxin
VN_1/VN_2	putative rat hindlimb vanilloid receptors
VO_2	oxygen consumption
VR_1	cloned dorsal root ganglion vanilloid receptor
Δ	change in

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ABSTRACT

Previous studies with the vanilloid spice principle capsaicin have shown a biphasic VO_2 response, with increased perfusion pressure (PP or vasoconstriction), in the perfused rat hindlimb that has led to suggestions of vanilloid receptor subtypes (VN_1/VN_2) in this preparation (Colquhoun *et al.*, 1995). The present study attempted to further define the role of vanilloid receptors by the use of selective competitive (capsazepine or CPZ) and non-competitive (ruthenium red) vanilloid antagonists. CPZ inhibited capsaicin-mediated effects in the perfused hindlimb in a competitive manner with an affinity, estimated by Schild plot analysis, comparable to that seen in other preparations. Submicromolar concentrations of CPZ selectively inhibited the increased VO_2 produced by the putative VN_1 receptor, and inhibited all effects at higher concentrations.. Submicromolar concentrations of ruthenium red, a vanilloid cation channel blocker, selectively inhibited the putative VN_2 receptor-mediated effects of capsaicin (strong vasoconstriction and inhibition of VO_2). These observations, showing different sensitivity to blockade by CPZ and ruthenium red, further support the presence of two vanilloid receptor/ion channel subtypes in the rat hindlimb.

Tetrodotoxin (TTX) failed to attenuate any changes produced by capsaicin, suggesting that the mechanism of action of capsaicin in the rat hindlimb may differ from other tissues where TTX-sensitive an insensitive cellular mechanisms operate. Moreover, 12-phenylacetate 13-acetate 20-homovanillate (PPAHV), shown to bind to vanilloid receptors in a non-cooperative manner, induced very similar effects to capsaicin and provided preliminary evidence that the biphasic effects of capsaicin on VO_2 are not likely to be due to cooperativity of binding to vanilloid receptors.

The role of capsaicin-sensitive neurons and their peptide neurotransmitters in capsaicin-induced responses in the perfused hindlimb were also studied. Non-peptide antagonists for tachykinin NK1 and NK2 receptors (CP-99,994 and SR 48968) selectively blocked the stimulation of VO_2 produced by submicromolar concentrations of capsaicin. Furthermore, infused substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) stimulated VO_2 and induced mild vasoconstriction with a rank order of potency NKA>NKB>SP. These data support a role for the tachykinins, acting primarily at NK2 receptors, in capsaicin-induced stimulation of VO_2. Infused calcitonin gene-related peptide (CGRP) did not alter basal VO_2 or PP. However, the CGRP

receptor antagonist $CGRP_{(8-37)}$ potentiated both VO_2 and PP responses to capsaicin in the rat hindlimb indicating a vasodilator role for endogenously released CGRP.

The stimulation of VO_2 and PP increase produced by low concentrations of infused capsaicin were absent one day after capsaicin pretreatment supporting a role for capsaicin-sensitive neurons in these responses. Conversely, the inhibition of VO_2 and increased PP produced by micromolar concentrations of capsaicin were potentiated 1, 7 and 14 days after capsaicin pretreatment and may either support a vasodilator role for endogenously released neuropeptides (e.g. CGRP), which are known to be depleted by capsaicin pretreatment, or upregulation of VN_2 receptors.

The vascular actions of CGRP, SP and NKA were further examined in the perfused rat hindlimb under noradrenalin (NOR)-induced vasoconstriction. CGRP caused strong, sustained dilatation that was nitric oxide (NO)-independent, while SP and NKA elicited transient dilatation that was partly NO-dependent. Conversely, a low concentration of capsaicin elicited strong vasoconstriction in the NA-stimulated perfused hindlimb. The metabolic effects of these agents was unclear from these studies given the strong VO_2-stimulating effect of NOR.

The studies undertaken support the notion that at least two vanilloid receptor subtypes mediate the vascular and metabolic effects of capsaicin in the perfused rat hindlimb. The effects of capsaicin in this tissue may result from a combination of dilatation and VO_2 stimulation induced by endogenous sensory neuropeptides, and the predominant vasoconstrictor effect of capsaicin via stimulation of specific vanilloid receptors. These findings underline the complex nature of capsaicin's novel actions in perfused rat skeletal muscle.

Chapter 1

Introduction

1.1. The Vanilloid Spice Principles

The consumption of spices as flavour enhancers has been common practice in many traditional communities for centuries, and is now increasingly widespread in 'western' societies. The ability of these foods to induce a local sensation of warmth can be attributed the presence of structurally similar pungent compounds including capsaicin (from chillies of the genus *Capsicum*) (Fig. 1.1), piperine (from black peppers), and gingerols and shogaols (from ginger root). Recently included in this class of naturally-occurring 'vanilloid' agents is the ultrapotent analogue resiniferatoxin (from the latex of some members of the genus *Euphorbia*) which is not only structurally similar to capsaicin (Fig. 1.1), but is also capsaicin-like in terms of its irritancy (Szallasi and Blumberg, 1989).

In the field of neuroscience, capsaicin and related compounds are best known for their ability to induce pain and inflammatory responses followed by a state of antinociception (desensitisation) in most mammalian species (Holzer, 1991). The sensitivity of these tissues to capsaicin and other vanilloid agents is typically due to the selective action of these compounds on capsaicin-sensitive primary afferent neurons (CSPANs) (see section *1.3* and sections therein) which have their cell bodies in the dorsal root ganglia of the spinal cord, and nerve endings in most body tissues/organs (Holzer, 1988). CSPANs can be identified with a population of sensory neurons with small diameter somata and either unmyelinated (C-type) or thinly myelinated (Aδ-type) fibres, although these morphological characteristics are not exclusive to CSPANs and it is their capsaicin sensitivity that distinguishes them from other primary afferent neurons (Holzer, 1991; Maggi, 1993). In addition to the transmission of nociceptive information to the CNS (afferent function) CSPANs also have a local efferent function usually involving the initiation of inflammatory events (neurogenic inflammation) mediated by the secondary release various neuropeptides. These include, most notably, the

tachykinins – substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) - and calcitonin gene-related peptide (CGRP) (Buck and Burks, 1986; Maggi and Meli,

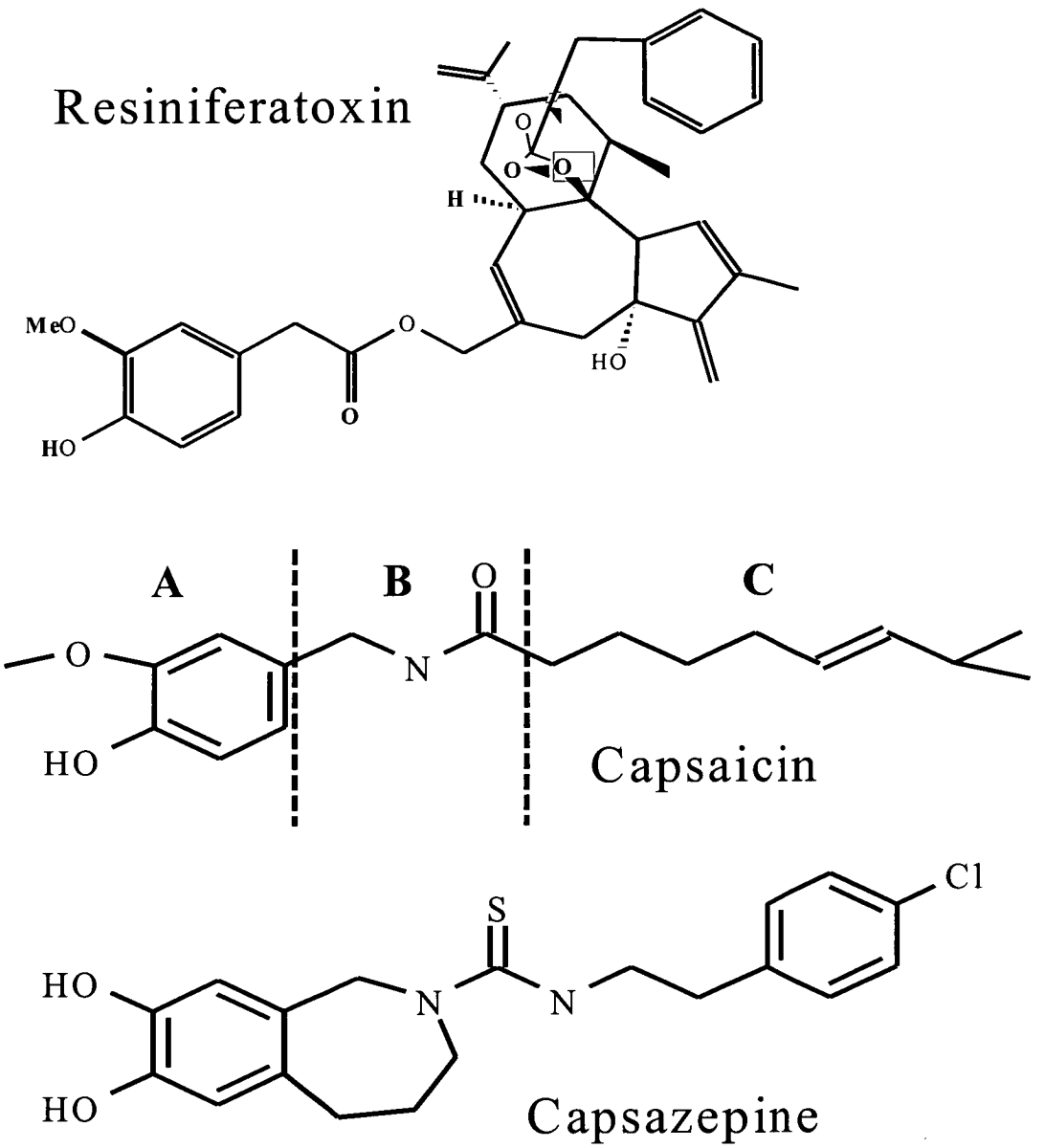


Fig. 1.1. The chemical structures of capsaicin, the ultrapotent analogue resiniferatoxin, and the competitive antagonist capsazepine. A commonly adopted approach in vanilloid structure-activity considerations is to divide the molecule into three regions: A (a 4-hydroxy, 3-methoxy aromatic ring); B (an amide 'linker'); and C (a hydrophobic sidechain), as shown for capsaicin.

1988; Holzer, 1988). The sensory efferent functions of CSPANs are well characterised in many smooth muscle organs including the lung (Lou, 1993; Lundberg, 1993), urinary bladder (Maggi, 1992), and vasculature (Holzer, 1993; Holzer, 1998) where release of

their peptide neuromodulators tends to elicit smooth muscle contraction, inflammation, and endothelium-dependent vasodilatation by stimulation of their respective receptors (Otsuka and Yoshioka, 1993; Bell and McDermott, 1996).

1.2. The Perfused Rat Hindlimb and the Metabolic Actions of Vanilloids

1.2.1. Use of the perfused rat hindlimb for metabolic studies in skeletal muscle

In studies exploring the factors controlling skeletal muscle metabolism, two techniques have been favoured; isolated and incubated (or perifused) muscle preparations, and the perfused rat hindlimb (hindquarter) preparation (reviewed by Bonen *et al.*, 1994). While incubated muscle techniques have been popular in the past, due largely to their simplicity, perfused rat hindlimb preparations offer the advantage of having nutrient supply by their own vascular networks, rather than relying on simple diffusion to parenchymal tissue. This latter consideration is particularly important in view of the evidence supporting a role for the vasculature in many of the metabolic responses in muscle (reviewed by Clark *et al.*, 1995; 1997).

Since the early report by Ruderman *et al.* (1971) describing a stable technique for rat hindlimb perfusion, use of the preparation has become widespread. The technique has been central to a variety of skeletal muscle metabolic studies including those into carbohydrate metabolism (Ploug *et al.*, 1987); oxygen consumption (VO_2 – an indirect measure of thermogenesis) (Grubb and Folk, 1976; 1977); exercise physiology (Côté *et al.*, 1985), vascular properties (Rippe *et al.*, 1978) and pharmacological studies (Kobinger and Pichler, 1981; Ye *et al.*, 1995; Hall *et al.*, 1997). In fact, the use of perfused hindlimb preparations has contributed significantly to a growing body of evidence that the vasculature plays a key role in regulating the metabolic behaviour of skeletal muscle (Clark *et al.*, 1995; 1997). Several reports have demonstrated the ability of vasoconstrictors (termed 'type A' vasoconstrictors) to increase hindlimb VO_2 (reduce venous PO_2), including catecholamines (Grubb and Folk, 1977; Richter *et al.*, 1982; Côté *et al.*, 1985; Colquhoun *et al.*, 1990; Dora *et al.*, 1992), peptides such as angiotensin II and vasopressin (Colquhoun *et al.*, 1988) and a number of spice principles (see section *1.2.3*). Furthermore, these agents have been shown to increase the efflux of other metabolic indicators including lactate, glycerol, and purine and pyrimidine metabolites (Clark *et al.*, 1995). Conversely, the net inhibition of hindlimb

VO_2 (increased venous PO_2) has been observed with the infusion of other vasoconstrictors (termed 'type B' vasoconstrictors) such as 5-hydroxytryptamine (5-HT or serotonin) (Dora *et al.*, 1991; 1992), high concentrations of noradrenalin (NOR) (Grubb and Folk, 1976; Côté *et al.*, 1985; Dora *et al.* ,1992) and high dose vanilloids (section *1.2.3.*).

1.2.2. Proposed mechanisms for vasoconstrictor-induced alteration of muscle metabolism

1.2.2.1. Working (contracting) vascular smooth muscle ('Hot Pipes')

The metabolic and vascular effects of type A vasoconstrictors, and to a slightly lesser extent type B vasoconstrictors, in the constant flow perfused rat hindlimb appear to be closely linked since all previous attempts to separate the two using nitrovasodilators (Ye *et al.*, 1990; Colquhoun *et al.*, 1990; Rattigan *et al.*, 1993), Ca^{2+} channel blockers (Colquhoun *et al.*, 1990), or metabolic poisons (Dora *et al.*, 1992; Richards *et al.*, 1992) were unsuccessful. The close association between vasoconstriction and oxygen uptake lead to the original proposal that stimulation of metabolism (including VO_2) by type A vasoconstrictors was largely the result of working (contracting) vascular smooth muscle ('hot pipes') (reviewed by Colquhoun and Clark, 1991). However, based on this model alone, it is difficult to account for the negative metabolic effects of strong vasoconstrictors such as serotonin. Furthermore, the efficacy of type A vasoconstrictors for increasing perfusion pressure does not show a simple direct relationship to their ability for increasing VO_2 (Clark *et al.*, 1995). Nonetheless, experiments using the isolated rat tail artery preparation have demonstrated the presence of sufficient mitochondrial and cytochrome oxidase activity for a potentially significant thermogenic contribution by this tissue (Clark *et al.*, 1995).

1.2.2.2. Nutritive and non-nutritive flow patterns

Of the possible explanations to account for the metabolic actions of both type A and type B vasoconstrictors in the constant flow perfused hindlimb, the currently favoured hypothesis involves the stimulation of site-specific receptors promoting flow redistribution within this tissue (Clark *et al.*, 1995; 1997). That is, vasoconstrictors that increase hindlimb VO_2, and efflux of metabolic by-products, probably do so by

redistributing perfusate flow to the network of vessels supplying skeletal muscle cells, resulting in greater total nutrient exchange. Indeed, there is a growing body of evidence from Clark's group in particular that the proposed increase in 'nutritive' flow in response to type A vasoconstrictors is responsible for increased resting metabolism in this tissue. This evidence includes: improved aerobic muscle contraction coincides with the infusion of type A vasoconstrictors, while type B vasoconstrictors inhibit contraction (Dora *et al.*, 1994; Rattigan *et al.*, 1996); in the rat hindlimb perfused at constant flow with erythrocyte-free medium, type A vasoconstrictors elicit a wash-out of red blood cells from previously under-perfused regions of the tissue (Newman *et al.*, 1996); FITC-labelled dextran can be entrapped in and released from the hindlimb by low-dose NOR suggesting the recruitment of a separate vascular network by this agent (Newman *et al.*, 1996); type A concentrations of NOR increase the conversion of 1-methyl xanthine to 1-methyl urate by the capillary enzyme xanthine oxidase, while the type B vasoconstrictor serotonin decreases conversion (Clark *et al.*, 1997). Recent evidence has also indicated that in addition to promoting a redistribution of perfusate flow, type A vasoconstrictors may stimulate resting muscle metabolism by a mechanism involving the activation of voltage-dependent Na^+ channels, possibly by the release of a veratridine-like substance (Tong *et al.*, 1998). This evidence coincides with the earlier suggestion that the metabolic actions of type A vasoconstrictors are likely to involve the release of an endogenous paracrine or autocrine signal (Clark *et al.*, 1995; 1997).

There is also convincing evidence that strong vasoconstrictors that reduce VO_2 in the perfused hindlimb do so by shunting perfusate away from nutritive vessels to so-called 'non-nutritive' vessels in this tissue (Clark *et al.*, 1995; 1997). Recent evidence from surface fluorometry experiments measuring FITC-dextran flow on the tendon of the biceps femoris, in response to serotonin, suggests that these vessels may supply hindlimb connective tissue such as the septa and tendons (Newman *et al.*, 1997). These authors propose that these vessels may represent a functional vascular shunt in this tissue.

1.2.3. Vanilloid actions in the perfused rat hindlimb

The serendipitous discovery that submicromolar concentrations of capsaicin and dihydrocapsaicin, the active pungent ingredients in chilli, could increase resting

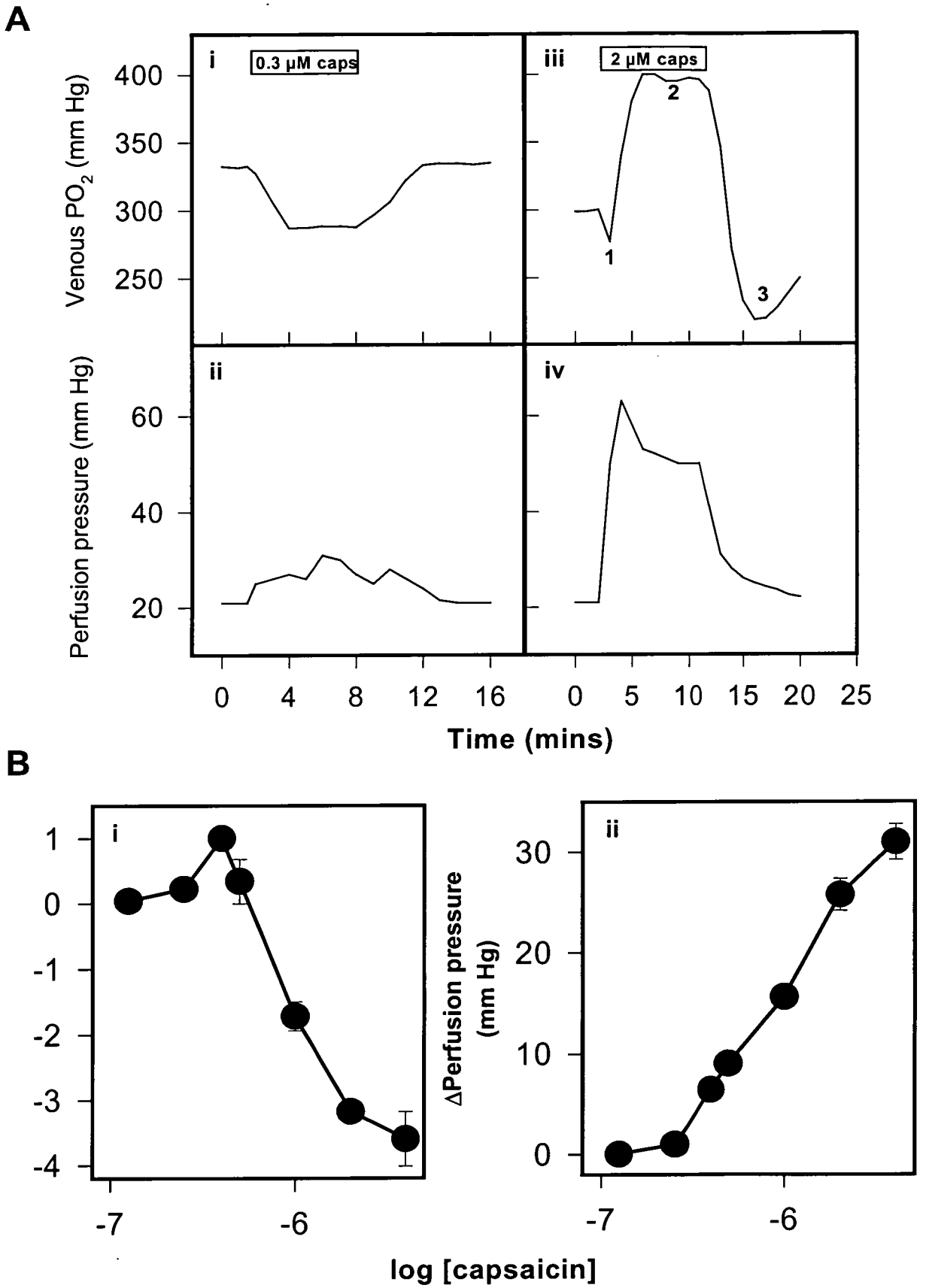

A
i
0.3 µM caps
iii
2 µM caps
1
2
3
ii
iv
Venous PO_2 (mm Hg)
400
350
300
250
Perfusion pressure (mm Hg)
60
40
20
0
4
8
12
16
0
5
10
15
20
25
Time (mins)
B
i
ii
ΔVO_2 (µmol. $g.^{-1}$ h^{-1})
1
0
-1
-2
-3
-4
ΔPerfusion pressure (mm Hg)
30
20
10
0
-7
-6
-7
-6
log [capsaicin]

Fig. 1.2. The effects of capsaicin on perfusion pressure and venous PO_2 (and hence VO_2) in the perfused rat hindlimb. A) Typical tracings of the effects of low (submicromolar) (i and ii) and high (micromolar) (iii and iv) capsaicin concentrations on venous PO_2 (i and iii) and arterial perfusion pressure (ii and iv) in the perfused rat hindlimb. Low concentrations of infused capsaicin induce a sustained steady state reduction in venous PO_2 (increase VO_2) (i) and mild sustained increase in perfusion pressure (ii) indicating increased vascular resistance (vasoconstriction). Removal of the vanilloid results in return of venous PO_2 and perfusion pressure to basal (unstimulated) levels. The effects of high capsaicin concentrations on venous PO_2 are triphasic (iii). Infusion of capsaicin causes an initial transient reduction in venous PO_2 (phase 1) followed by a rapid increase in venous PO_2 to a steady state value above basal venous PO_2 (phase 2) (inhibition of VO_2). Finally, the removal of capsaicin results in a second, but stronger, transient decrease in venous PO_2 (phase 3) before recovery to basal. High capsaicin concentrations also elicit a strong, sustained increase in arterial perfusion pressure (iv). B) Concentration-response curves for capsaicin induced changes in VO_2 (i) and perfusion pressure (ii). Venous PO_2 (VO_2) and perfusion pressure values for each concentration of capsaicin are taken from the steady state phase of the responses shown in A).

hindlimb VO_2 (reduce venous PO_2) revealed these compounds as an exciting prospect for their use as *in vivo* metabolic stimulants (Cameron-Smith *et al.*, 1990). Findings very similar to those seen in this study are presented in Fig 1.2, with the inclusion of the negative metabolic effects observed at micromolar concentrations of capsaicin. Other naturally occurring members of the vanilloid class of molecules, such as gingerols and shogaols (Eldershaw *et al.*, 1992), and resiniferatoxin (RTX) and piperine (Eldershaw *et al.*, 1994), were shown to produce qualitatively and quantitatively similar effects on hindlimb VO_2. It was also revealed in these studies that in addition to stimulating VO_2 at low effective concentrations, all of these compounds induced a steady state reduction in hindlimb VO_2 (increase venous PO_2) at high effective concentrations. Further analysis of the effects of capsaicin showed a very similar biphasic effect on resting hindlimb VO_2 (Colquhoun *et al.*, 1995). Interestingly, the cessation of exposure to high

effective concentrations of vanilloids results in a transient stimulation of VO_2 (reduction in venous PO_2) (Fig. 1.2Aiii) before recovery to basal values (not shown).

Associated with the biphasic VO_2 effect of all vanilloid analogues is a concentration-dependent increase in perfusion pressure, indicating increased vascular resistance and, therefore, constriction of the hindlimb vasculature (Fig 1.2). As for other vasoconstrictors, the vascular and metabolic effects of vanilloids seem to be linked since nitrovasodilators were able to inhibit both responses (Cameron-Smith *et al.*, 1990; Eldershaw *et al.*, 1992; 1994). Furthermore, several vanilloid agents have been shown to induce a biphasic effect on lactate efflux where lactate is increased at low effective concentrations, and reduced at higher effective concentrations (Eldershaw *et al.*, 1992; Colquhoun *et al.*, 1995).

The mechanisms by which vanilloids induce these effects in the perfused rat hindlimb were not clear from these initial studies. Unlike the effects seen on whole body VO_2 (see section *1.2.4.*), the actions of capsaicin do not appear to involve the secondary release of catecholamines given the failure of adrenergic blockers to inhibit capsaicin-mediated changes in VO_2 or vascular resistance (vascular tension) (Cameron-Smith *et al.*, 1990). It is apparent, however, that the effects induced by low and high effective vanilloid concentrations have different metabolic requirements, including a differing dependence on extracellular calcium (Colquhoun *et al.*, 1995). Based on these findings, these authors propose that the mechanisms of capsaicin action in the perfused rat hindlimb may differ at low and high concentrations, possibly involving the stimulation of different vanilloid receptor subtypes.

1.2.4. In vivo thermogenic actions of vanilloids

While the thermogenic effect of vanilloids on skeletal muscle metabolism may represent a potentially interesting way to induce whole-body energy expenditure and weight loss, there are few reports describing the metabolic effects of these agents *in vivo*. Some indirect evidence was obtained in the study of Henry and Emery (1986) who noted a marked increase (approximately 25%) in whole-body oxygen consumption (thermogenesis) in 12 healthy human subjects fed a pungent mixture of chilli and mustard sauces. This thermogenic effect was sustained for at least 180 minutes (post-meal measurement period), however it is unclear whether or not the effect was caused specifically by vanilloids, or other ingredients contained in the sauces. Indeed, a later

study using a similar dietary treatment showed that a spicy meal did not significantly alter whole-body oxygen consumption, but did lead to sleep disturbances which may have been indicative of a thermoregulatory effect (Edwards *et al.*, 1992).

Studies on the metabolic effects of vanilloids in the rat have been more conclusive. The intraperitoneal injection of capsaicin (Kawada *et al.*, 1986) or piperine (Kawada *et al.*, 1988), at doses calculated to occur in a highly spiced diet, increased oxygen consumption in these animals. However, the effects of vanilloids on energy metabolism in rats is likely to be due to the stimulation of catecholamine secretion by these agents (Watanabe *et al.* ,1987; Kawada et al., 1988) via activation of the CNS by CSPAN impulses (Watanabe *et al.*, 1991). Indeed, the *in vivo* oxygen consumption response to capsaicin was shown to be sensitive to the β-adrenergic blocker propranolol (Kawada *et al.*, 1986).

1.3. Aspects of Vanilloid Actions on Sensory Neurons

The mechanism by which vanilloid spice principles elicit their effects on a variety of target tissues has been the subject of extensive reviews (Holzer, 1991; Wood, 1993). Most studies on the actions of the vanilloids have been restricted largely to capsaicin and its excitatory, desensitising and toxic effects on CSPANs (Holzer, 1988; 1991; Szolcsanyi, 1993). The sensitivity of these neurones to capsaicin is likely to be due to the presence of a receptor-coupled cation channel that, when stimulated by vanilloids, allows the influx of various cations facilitating the release of several neuropeptide modulators (Holzer, 1991; Bevan and Szolcsanyi, 1990). Thus there is an ionic basis for the excitatory, desensitising and neurotoxic effects of capsaicin which is mediated by the stimulation of specific vanilloid recognition sites. Such 'cell-selective' effects of capsaicin are distinct from the 'non-selective' effects of this pungent spice principle (reviewed by Holzer, 1991, Szallasi, 1994) discussed in section *1.4.*

1.3.1. Evidence for the existence of vanilloid receptors

1.3.1.1. Historical aspects

The existence of a specific capsaicin receptor was first proposed by the Hungarian group of Szolcsanyi and Janscó-Gábor (1975; 1976) who observed a structural

relationship between various synthetic vanilloid analogues and their pain-producing activity. Since these early studies, substantial evidence has accumulated indicating that the actions of both capsaicin and the ultrapotent analogue resiniferatoxin (RTX) are mediated via specific vanilloid receptors that are believed to be related intimately with a capsaicin-operated cation channel (James *et al.*, 1993). With the recent cloning of a capsaicin receptor-channel complex from rat DRG cDNA (Caterina *et al.*, 1997), there is now compelling evidence that this initial hypothesis was correct. However, prior to this breakthrough there were other important lines of evidence in support of the existence of vanilloid recognition sites. Most notably, the discovery of capsazepine, the first competitive vanilloid antagonist (Dickenson and Dray, 1991; Urban and Dray, 1991) and the development of a radioligand binding assay (Szallasi and Blumberg, 1990a; 1990b) were important steps in the progression of vanilloid receptor pharmacology. To a lesser but important extent, studies using the functional, non-competitive antagonist ruthenium red have also contributed to our understanding of these novel binding sites (reviewed by Amman and Maggi, 1991).

1.3.1.2. Ruthenium red

Prior to the development of the competitive antagonist capsazepine, ruthenium red was the only known capsaicin antagonist. Preceding the discovery of this polycationic dye as a blocker of capsaicin-mediated responses (Wood *et al.*, 1988; Maggi *et al.*, 1988b), it was widely used in the 1960's as a pectin stain in plant physiology and subsequently as an inhibitor of calcium transport in mitochondria at high micromolar concentrations (Moore, 1971; Vasington *et al.*, 1972). Further testament to the ability of this compound to inhibit membrane calcium fluxes was obtained when it was discovered that millimolar concentrations of ruthenium red were able to inhibit smooth muscle contraction by blockade of voltage-sensitive calcium channels (Greenberg *et al.*, 1973). Subsequent studies in both smooth muscle and skeletal muscle have demonstrated that micromolar concentrations of the dye are able to inhibit caffeine-induced release of intracellular calcium stores from the sarcoplasmic reticulum in these tissues (Salviati and Volpe, 1988; Kanmura *et al.*, 1989).

In view of the ability of ruthenium red to induce such general inhibition of intracellular and extracellular calcium fluxes, it is reasonable to assume that this may be related to its ability to block capsaicin-mediated responses. However, in a large number

of preparations, ruthenium red has been shown to selectively block the excitatory actions of capsaicin without blocking the effects of other stimuli (reviewed by Amann and Maggi, 1991), as well as blocking the unique desensitising properties of this pungent principle (Chahl, 1989; Amann *et al.*, 1990). For example, measurements of calcium influx into rat DRG neurons showed that ruthenium red (100 nM) was able to inhibit capsaicin-induced increases in intracellular calcium (measured using the calcium sensitive fluophore Fura-2), but not those induced by bradykinin (Dray *et al.*, 1990a). Using patch-clamp techniques, these authors also demonstrated the ability of low concentrations of ruthenium red to selectively inhibit capsaicin-activated single ion channel currents in rat DRG neurons, and concluded from the pattern of the blockade that it was occurring at a site distinct from the channel itself. Thus, although the mechanism of ruthenium red inhibition of the capsaicin-operated channel is not fully understood, it is likely to involve impairment of the receptor-channel coupling mechanism (Dray *et al.*, 1990a). It is unlikely that ruthenium red blocks the binding of capsaicin to its receptor by a competitive interaction given that the dye was unable to displace the specific binding of [^{3}H]-RTX to rat DRG membranes (Szallasi and Blumberg, 1990a). Furthermore, ruthenium red caused a characteristic non-competitive antagonism of capsaicin-mediated effects in cultured rat DRG neurons (Bevan *et al.*, 1992) and in rat urinary bladder and *vas deferens* (Maggi *et al.*, 1993a), that was distinct from the competitive inhibition produced by capsazepine in these studies.

Ruthenium red represents a useful pharmacological tool for the study of capsaicin-operated cation channels at submicromolar to micromolar concentrations with higher concentrations (usually >1 μM) resulting in the loss of selectivity for the capsaicin receptor cation channel complex (reviewed by Amann and Maggi, 1991). Nonetheless, moderately high micromolar concentrations (10 μM) of the dye have still demonstrated selectivity for the actions of capsaicin, including the release of CGRP from the perfused guinea-pig lung (Amann *et al.*, 1989) and capsaicin-evoked relaxation of human isolated ileum (Giuliani *et al.*, 1991). Further confirmation of the ability of this 'functional' antagonist to selectively block the capsaicin ligand-gated channel was obtained recently in whole-cell patch clamp studies where ruthenium red completely attenuated the inward current evoked by capsaicin in *Xenopus* oocytes expressing cloned vanilloid receptors (Caterina *et al.*, 1997).

1.3.1.3. Capsazepine

At the time of its development, capsazepine provided one of few useful pharmacological tools to characterise the little known vanilloid receptor. Structurally, capsazepine is similar to capsaicin (Fig. 1.1) with three major changes present on the antagonist: a) the methoxy group on position 3 of the aromatic ring (A-region) of capsaicin has been substituted for a hydroxyl group creating a 3,4-dihydroxy phenyl ring, permitting ease of synthesis (Walpole and Wrigglesworth, 1993); b) the amide 'linker' region (B-region) of capsaicin has been replaced by a thiosemicarbazide in capsazepine; and c) replacing the long hydrophobic sidechain of capsaicin (C-region) is a 4-chlorophenylethyl side chain in capsazepine. From the conformational analysis of capsazepine, with respect to agonist structures, it has been proposed that the pharmacological change from agonist to antagonist is likely to be due to constraints placed on the A- and B-regions by the presence of the seven-membered saturated ring in capsazepine (Walpole *et al.*, 1994). Thus, capsazepine adopts a 'pseudochair' conformation where the plane of the B- and C-regions are held approximately perpendicular to the aromatic ring.

The first compelling evidence that capsazepine competes directly for the vanilloid (capsaicin) receptor came with the study of Bevan *et al.* (1992) who demonstrated the ability of this compound to inhibit RTX-induced $^{45}Ca^{2+}$ uptake by cultured rat dorsal root ganglion (DRG) neurons, capsaicin- and RTX-induced $^{86}Rb^{+}$ efflux by DRG neurons, and capsaicin-induced [^{14}C]-guanidinium efflux from rat vagus nerves. In all studies, increasing the capsazepine concentration caused a parallel shift to the right in the concentration-response curves for capsaicin and RTX, without reducing the maximal response to either agonist. Supporting the apparent competitive nature of this inhibition was the Schild analysis which yielded slopes close to unity (where slopes equal to 1 are indicative of a competitive relationship between agonist and antagonist). In addition, capsazepine failed to alter the effects of other depolarising stimuli (high potassium concentrations, low pH, GABA, ATP), at concentrations capable of almost completely blocking the effects of capsaicin and RTX, confirming its selectivity for the vanilloid binding site.

Maggi and his co-workers showed a similar ability of capsazepine to competitively inhibit the actions of capsaicin at peripheral vanilloid receptors in the rat urinary bladder and vas deferens (Maggi *et al.*, 1993). Concentration-response curves

for capsaicin-induced urinary bladder contraction and inhibition of electrically-evoked twitches of the *vas deferens* showed a parallel shift to the right, without a reduction of the maximal response, in the presence of 3-30 μM capsazepine. Capsazepine failed to inhibit post-junctional stimulation of these tissues by NKA (bladder) and CGRP (*vas deferens*) again indicating selective blockade of a prejunctional vanilloid receptor.

Since these early pioneering studies, capsazepine has been used to block the actions of vanilloids in functional studies in numerous tissues including rat lung (Lou and Lundberg, 1992; Lee and Lundberg, 1994), *vas deferens* (Wardle *et al.*, 1996), skeletal muscle (Santicioli *et al.*, 1993), knee joint (Davis and Perkins, 1996), and skin (Seno and Dray, 1993), as well as guinea pig airways (Belvisi *et al.*, 1992; Ellis and Undem, 1994) and heart (Franco-Cereceda *et al.*, 1993). In addition, capsazepine was shown to inhibit the binding of [^{3}H]-RTX to rat spinal cord vanilloid receptors without reducing the maximum binding (B_{max}) of the radioligand (Szallasi *et al.*, 1993c). Other radioligand binding studies have shown capsazepine to inhibit [^{3}H]-RTX binding to vanilloid receptors in membrane preparations from rat DRG (Szallasi *et al.*, 1993c; Acs *et al.*, 1994b, 1996), lung (Szallasi *et al.*, 1993c), colon (Goso *et al.*, 1993b), urinary bladder (Szallasi *et al.*, 1993c; Acs *et al.*, 1994b) and sciatic nerve (Acs *et al.*, 1994b), as well as membrane preparations the dorsal horn of human spinal cord (Acs *et al.*, 1994a). More recently, capsazepine was shown to block capsaicin- and RTX-induced calcium influx into *Xenopus* oocytes and human HEK cells expressing cloned vanilloid receptors (Caterina *et al.*, 1997).

While the above evidence is quite compelling, the uncontrolled use of capsazepine should be avoided given that, inevitably, some studies question the spectrum of actions this compound and its selectivity for vanilloid receptors at high concentrations. In the rabbit iris sphincter both capsazepine and ruthenium red, at concentrations up to 100 μM, not only inhibit the constriction induced by capsaicin but also that induced by bradykinin, NKA and electrical field stimulation (Wang and Håkanson, 1993). In addition, capsazepine and ruthenium red act as partial agonists in this preparation. Furthermore, micromolar concentrations of capsazepine were recently shown to produce slowly developing, but irreversible, inhibition of voltage-activated calcium channels in cultured rat DRG (Docherty *et al.*, 1997) and *Xenopus* embryo spinal neurons (Kuenzi and Dale, 1996). Docherty *et al.* (1997) propose that this observation may account for capsazepine's blockade of neuropeptide release induced by non-vanilloid stimuli such as

protons (eg. Franco-Cereceda and Lundberg, 1992; Franco-Cereceda *et al.* 1993; Santicioli *et al.*, 1993), rather than the release of an endogenous vanilloid-like mediator. Of greater concern is the ability of 10 μM capsazepine to reversibly inhibit the currents induced by 100μM nicotine in rat trigemminal ganglia suggesting that the antagonist may bind to other ligand-gated channels (nicotinic acetylcholine receptors) distinct from the vanilloid channel (Liu and Simon, 1997).

1.3.1.4. Radiolabelled resiniferatoxin ([^{3}H]-RTX)

Attempts in the 1980's to demonstrate the existence of capsaicin (vanilloid) receptors by the use of radiolabelled dihydrocapsaicin (Miller *et al.*, 1982) or a capsaicin-like photoaffinity probes (James *et al.*,. 1988) were unsuccessful due to the relatively poor potency and high lipophilicity of these compounds. During this time, an important step in the development of a radiolabelled ligand for the identification of vanilloid receptors was the discovery that a naturally occurring phorbol ester, isolated from the latex of *Eurphobia spp.*, was an ultrapotent capsaicin analogue (deVries and Blumberg, 1989; Szallasi and Blumberg, 1989). From the appropriately named resiniferatoxin (RTX) (Fig. 1.1), a radiolabelled (tritiated) analogue ([^{3}H]-RTX) was developed resulting in the first studies identifying vanilloid receptors in radioligand binding studies using rat DRG membranes (Szallasi and Blumberg, 1990a; 1990b), and subsequently by autoradiography (Szallasi *et al.*, 1994; Szallasi, 1995; Szallasi *et al.*, 1995). Vanilloid receptors have since been identified in several species (reviewed by Szallasi, 1994), including man (Acs *et al.*, 1994a; Acs *et al.*, 1996), but not in species that are insensitive to vanilloids, such as the chicken (Szallasi and Blumberg, 1990b).

Not only was conclusive proof obtained for the existence of the long-sought vanilloid receptor, the [^{3}H]-RTX binding assay has also revealed important information on the function of this novel binding site. The binding of [^{3}H]-RTX to vanilloid receptors was shown to have marked temperature dependence and also showed sensitivity to sulfhydryl-reactive agents, indicating that the receptor is dependent on free sulfhydryl groups for agonist binding (Szallasi and Blumberg, 1993). Furthermore, numerous studies have now shown that the binding of [^{3}H]-RTX to membrane preparations from several sources can be selectively inhibited by capsazepine (see sections *1.3.1.3 and 1.3.2.1*) and various other natural and synthetic vanilloid analogues (Szallasi and Blumberg, 1990a; 1990b; Szallasi *et al.*, 1993a; 1993b; 1993c; Acs and

Blumberg, 1994a; 1994b; Acs *et al.*, 1994b; Acs *et al.*, 1996; Szallasi *et al.*, 1996) indicating the presence of a common binding site for these ligands.

While the first studies using the [^{3}H]-RTX binding assay were successful, several methodological limitations were apparent, most of which related to the high level of non-specific binding (approximately 50%) seen with the use of this extremely lipophilic molecule (Szallasi and Blumberg, 1990a). These limitations made it difficult to analyse [^{3}H]-RTX binding at low or high concentrations, or to identify vanilloid receptors in the spinal cord and peripheral tissues (reviewed by Szallasi, 1994). Improvement in the [^{3}H]-RTX binding assay came with the discovery that the serum protein α_1-acid glycoprotein (orosomucoid) could bind RTX with micromolar affinity that is many times lower than the affinity of [^{3}H]-RTX for its receptors, and could bind RTX at 0°C where the association and dissociation of RTX and vanilloid receptors is immeasurably slow (Szallasi *et al.*, 1992). Hence the addition of α_1-acid glycoprotein at the termination of the [^{3}H]-RTX binding reaction (i.e. by cooling the assay mixture on ice) resulted in a substantial reduction in non-specific binding to approximately 10% of total binding (Szallasi *et al.*, 1992; Szallasi *et al.* 1993b, Acs and Blumberg, 1994b). This improvement in the [^{3}H]-RTX binding assay permitted, for the first time, the analysis of binding at low [^{3}H]-RTX concentrations (low fractional receptor occupancy) and revealed sigmoidal binding kinetics in rat DRG and spinal cord preparations that was indicative of cooperativity of binding (Szallasi *et al.*, 1993b). Furthermore, the cooperativity indices, estimated by Hill slope analysis, and curved (bell-shaped) Scatchard plots derived from these experiments were indicative of positive cooperativity. Additional evidence for the presence of an allosteric interaction was obtained by the observation that at low receptor occupancies, the binding of [^{3}H]-RTX showed marked enhancement then inhibition with the addition of increasing concentrations of non-radioactive RTX or capsaicin (i.e. biphasic competition curves) (Szallasi *et al.*, 1993b, Acs and Blumberg, 1994a; 1994b; Acs *et al.*, 1994).

The improvements in the [^{3}H]-RTX assay also permitted the characterisation of vanilloid receptors in peripheral tissues including the urinary bladder (Szallasi *et al.*, 1993a; 1993c), urethra (Parlani *et al.*, 1993), airways (Szallasi *et al.*, 1993c) and gut (Goso *et al.*, 1993). These studies have revealed an interesting distinction between peripheral receptors and those of the CNS with regard to the nature of agonist binding

and have, in part, contributed to the evidence for the existence of vanilloid receptor subtypes (see section *1.3.2*).

1.3.1.5. Molecular cloning strategies

In conjunction with functional and radioligand binding and/or autoradiographic studies, an important step in the characterisation of a receptor type or family is the identification and cloning of the gene encoding a particular binding protein. Until recently, strategies for the cloning of vanilloid receptors were unsuccessful, due largely to the inability to detect expressed vanilloid binding proteins and the lack of information regarding their molecular structure. The use of an RTX photoaffinity label (RTX-PAL) for the detection of RTX binding proteins in bacterial systems expressing transcripts of DRG cDNA has been moderately successful yielding several candidate clones (James *et al.*, 1993). However, analysis of the tissue distribution of mRNA for these clones was not consistent with the typical tissue distribution exhibited by sensory neuron membrane-associated receptors (James *et al.*, 1993).

The recent successful cloning of the vanilloid receptor was based on the well-known ability of capsaicin to activate ion channels resulting in the intracellular accumulation of calcium, and the detection of calcium build-up by microscopic fluorescence imaging (Caterina *et al.*, 1997). This innovative functional screening strategy was adopted for the isolation of candidate cDNA clones expressed in a non-neuronal mammalian cell line, and did not rely on the binding of a labelled ligand to poorly understood vanilloid binding sites. A cDNA library was constructed from rat DRG neuron mRNA and subsequently subdivided into large pools each containing approximately 16,000 clones. Each pool of clones was transiently transfected into human embryonic kidney derived HEK293 cells loaded with the fluorescent calcium-sensitive dye Fura-2, and the cells were then microscopically examined for changes in intracellular calcium in response to capsaicin exposure. Using this functional assay technique, a positive pool of clones was identified, subdivided and reassayed until a single 3-kilobase cDNA insert conferring capsaicin and resiniferatoxin sensitivity was obtained. This cloned cDNA was aptly named VR1 for vanilloid receptor subtype 1 (Caterina *et al.*, 1997).

Despite the fact that the cells expressing the VR1 cDNA insert in the study of Caterina et al. (1997) were non-neuronal cells, they still exhibited the 'classical' cellular

responses to capsaicin and resiniferatoxin that are ordinarily exclusive to small diameter neurons expressing native vanilloid receptors (reviewed by Bevan and Docherty, 1993). Whole-cell voltage clamp analysis of *Xenopus* oocytes expressing VR1 showed that at negative holding potentials both capsaicin and resiniferatoxin evoked a concentration-dependent inward current that was completely blocked by capsazepine (10 μM) or ruthenium red (10 μM) (Caterina *et al.*, 1997). This study also showed that at positive holding potentials, vanilloid agonists evoked an outward current with the reversal potential close to 0 mV, an observation that is consistent with the activation of a cation channel (Bevan and Forbes, 1988). Furthermore, in ion replacement studies, cells expressing VR1 did not discriminate between different cations when stimulated by capsaicin, but did show greater permeability to divalent cations (permeability sequence: $Ca^{2+} > Mg^{2+} > Na^{+} > K^{+} = Cs^{+}$), consistent with observations made in rat DRG neurons (Bevan and Docherty, 1993).

Analysis of the VR1 cDNA product revealed an 838 amino acid protein with a molecular mass of ~95K (Caterina *et al.*, 1997), a finding that is not consistent with earlier (but probably less accurate) estimates of vanilloid receptor size in pig DRG (~270K) (Szallasi and Blumberg, 1991). This discrepancy may also reflect the proposed species heterogeneity of vanilloid receptors (Szallasi, 1994). Interestingly, the deduced amino acid sequence of VR1 showed a high degree of homology with store-operated calcium channels (SOCs) that are believed to be involved in the regulation of intracellular calcium stores (Clapham, 1997), although Caterina *et al.* (1997) could not ascribe an SOC function to VR1. Indeed it is more likely that these receptors are involved in the perception of thermal stimuli given the ability of noxious heat to activate cation fluxes in both HEK293 cells and *Xenopus* oocytes expressing VR1 (Caterina *et al.*, 1997).

1.3.2. Vanilloid receptor subtypes

There is now mounting evidence that vanilloid recognition sites not only represent a novel type of 'pain' receptor, but that they may also represent an entire new receptor family. Indeed, the concept of vanilloid receptor subtypes has recently been the central topic of some excellent review articles (Szallasi, 1994; Szallasi and Blumberg, 1996; Appendino and Szallasi, 1997). Several convincing, albeit indirect, lines of evidence have now emerged in favour of vanilloid receptor heterogeneity, some of which are

discussed below and others, including that obtained in the perfused rat hindlimb, are outlined later in Chapter 2.

1.3.2.1. Evidence from [^{3}H]-RTX binding studies

Radioligand binding studies using [^{3}H]-RTX not only permitted the biochemical characterisation of the vanilloid receptor (Szallasi and Blumberg, 1990a; 1990b; 1993) but also demonstrated species heterogeneity of receptor binding, as well as possible intraspecies receptor subtypes (Szallasi, 1994). Peripheral vanilloid receptors in the urinary bladder (Szallasi *et al.*, 1993a), colon (Goso *et al.*, 1993b), and urethra (Parlani *et al.*, 1993) of the rat all bind [^{3}H]-RTX in a non-cooperative fashion, and show decreased affinity for RTX, that is distinct from the positive cooperativity of [^{3}H]-RTX binding central receptors (Szallasi and Blumberg, 1993; Szallasi *et al.*, 1993a; 1993b). In addition, capsaicin inhibits specific [^{3}H]-RTX binding to central vanilloid receptors with a 7 to 10-fold higher affinity than the competitive antagonist capsazepine (Goso *et al.*, 1993a), while at peripheral receptors in the colon, the order of potency is reversed (Goso *et al.*, 1993b). However, the binding behaviour of [^{3}H]-RTX and affinity of capsazepine for central and peripheral vanilloid receptors is not as clear-cut as the above studies indicate. A recent study did not show any major differences between the binding behaviour (i.e. cooperative vs. non-cooperative) of [^{3}H]-RTX to vanilloid receptors in CNS and peripheral tissues in the same species, nor was there any difference in the affinity of capsazepine for receptors in these tissues (Acs *et al.*, 1994b). The apparent discrepancy between the positive cooperativity of [^{3}H]-RTX binding in the urinary bladder in this study, and the non-cooperative nature of [^{3}H]-RTX binding in the same tissue in the study of Szallasi *et al.* (1993a) is not clear at present. Different assay conditions between the studies (eg. temperature and buffer composition), has been cited as a potential source of this discrepancy (Acs *et al.*, 1994b).

1.3.2.2. Evidence from the study of vanilloid-induced ion currents in vitro

The work of Simon and his colleagues has provided evidence for CNS vanilloid receptor subtypes in patch-clamped studies in rat trigeminal (Liu and Simon 1994; 1996) and DRG (Liu *et al.*, 1996) neurons. Most, but not all, of these neurons exhibit two types of inward current in response to capsaicin - one fast and one slow activating/inactivating - both of which are sensitive to capsazepine and ruthenium red

(Liu and Simon, 1994). Other neurons responded to capsaicin by the activation of a rapid inward current only, while RTX stimulates only slowly-activating currents (Liu and Simon, 1994; 1996). This latter finding was consistent with earlier findings for RTX actions on DRG neurons (Winter *et al.*, 1990) and may reflect the increased ability of this ultrapotent agent to induce desensitisation and cell death (Liu and Simon, 1996). Not only was there heterogeneity with respect to the currents activated by these pungent spice principles, but they also induced different degrees of desensitisation, with different calcium dependencies. This may reflect the presence of receptor/channel subtypes with different activation and desensitisation kinetics (Liu and Simon, 1996).

Further to these studies, this group recently presented evidence for the presence of receptor/channel subtypes on rat trigeminal ganglion neurons by use of the non-pungent RTX analogue phorbol 12-phenylacetete 13-acetate 20-homovanillate (PPAHV) (Liu *et al.*, 1998). PPAHV evoked three types of inward current in some trigeminal neurons that displayed different reversal potentials when current-voltage curves were constructed. Currents induced by PPAHV were kinetically distinct from the two inward currents evoked by capsaicin, however two of these showed very similar reversal potentials to those of capsaicin. In addition, repeated application of PPAHV prevented the rapidly-activating currents but only slightly reduced the slowly-activating current indicating that the different current types were separable. Furthermore, a high concentration of capsazepine (10 μM) failed to inhibit the inward currents activated by PPAHV in some, but not all, trigeminal neurons. Taken together, these findings indicate that there are likely to be receptor/ion channel subtypes that are activated by PPAHV with different activation and desensitisation kinetics, and that some of these complexes may represent a newly defined capsazepine-insensitive subtype of vanilloid receptor (Liu *et al.*, 1998).

1.3.2.3. Evidence from vanilloid structure-activity relationships

Structure-activity relations for receptor binding versus calcium uptake of some vanilloid analogues also provide indirect evidence for the existence of vanilloid receptor subtypes. RTX and related compounds (eg. tinyatoxin) are at least 10-fold more potent for binding vanilloid receptors than for inducing calcium uptake by sensory neurons (Acs *et al.*, 1996; Walpole *et al.*, 1996). However, the same studies showed that for capsaicin and its synthetic analogues, such as nuvanil, the reverse is apparent (i.e. they

are 10-fold more potent at inducing calcium uptake than for inhibiting [^{3}H]-RTX binding) suggesting that [^{3}H]-RTX binding and calcium uptake assays detect different types of vanilloid receptor (Szallasi and Blumberg, 1996).

At present there is no direct genetic evidence for the existence of vanilloid receptor subtypes, although such evidence may soon be obtained now that the molecular structure of a rat DRG vanilloid receptor is known (see section *1.3.1.5.*). The fact that VR1 mRNA could not be detected in several peripheral tissues (Caterina *et al.*, 1997) known to express [^{3}H]-RTX binding sites (Szallasi, 1994) may itself reflect the heterogeneity of these novel receptors.

1.3.3. Vanilloid cellular mechanisms

A number of studies, mostly on rat DRG neurons, have contributed to our understanding of how capsaicin and related compounds elicit their excitatory, desensitising and neurotoxic effects on CSPANs, and the ionic basis for these actions (reviewed by Holzer, 1991; Bevan and Docherty, 1993). Moreover, knowledge of the ion fluxes induced by vanilloids in sensory neurons has lead to better understanding of how these agents elicit the release of neuropeptide modulators from CSPANs (reviewed by Holzer, 1991; Maggi, 1993).

Microelectrode recordings on rat DRG neurons showed that the application capsaicin to the extracellular surface of these cells induces a rapid depolarisation in neurons with low axonal conductance velocities (C-type cells), but not fast-conducting neurons (A-type cells) (Heyman and Rang, 1985). This depolarisation is not sustained in the continued presence of capsaicin, and the membrane potential hyperpolarises back to negative values (Marsh *et al.*, 1987). Several lines of evidence from these and subsequent patch-clamp studies revealed that the initial depolarisation evoked by capsaicin involves the opening of cation channels in the membrane of CSPANs that are non-selective in terms of their permeability. Most notably: a) the depolarisation is accompanied by a decrease in cell input resistance; b) at positive holding potentials capsaicin and RTX evoke an outward current with the reversal potential for the inward current at 0 mV; and c) ion replacement studies show that the ion channel activated by capsaicin and RTX will permit the passage of different types of monovalent and divalent cations (Marsh *et al.*, 1987; Bevan and Forbes, 1988; Bevan and Szolcsanyi, 1990; Winter *et al.*, 1990). These findings were supported by radiotracer flux

experiments in rat DRG which showed that capsaicin and RTX could increase the uptake of various cations, as well as the efflux of other such as $^{86}Rb^{+}$ (used as an indicator of potassium flux) (Wood *et al.*, 1988; Winter *et al.*, 1990). Although non-selective, the vanilloid-operated cation channel does hold a preference for permitting the passage of divalent cations, such as calcium, with a permeability sequence $Ca^{2+} > Mg^{2+} > guanidinium^{+} > K^{+} > Na^{+} >$ choline (Bevan and Szolcsanyi, 1990; Bevan and Docherty, 1993). Indeed, recent studies with cloned vanilloid receptor-channel complexes indicate that they permit the passage of calcium and sodium in the ratio of approximately 10:1 in response to capsaicin (Caterina *et al.*, 1997, Clapham, 1997). Thus, capsaicin and RTX activate the same recognition site on CSPANs that leads to the opening of a channel that is permeable to different cations, particularly Ca^{2+}, and is distinct from voltage-sensitive Ca^{2+} channels (VSCCs) since blockers of several different VFCCs failed to inhibit the stimulant effect of capsaicin (Maggi *et al.*, 1988a; 1989; Wood *et al.*, 1988; Dray *et al.*, 1990b). In fact, the intracellular accumulation of Ca^{2+} in CSPANs leads to the inhibition of VSCCs, but promotes Ca^{2+}–dependent enzyme activation and, most importantly, neuropeptide release (Holzer, 1991).

1.3.4. Neuropeptide release

While capsaicin-induced generation of afferent impulses and local neuropeptide release (efferent function) both have ionic requirements, they differ in their dependence on external calcium. Afferent impulse discharge by capsaicin is not inhibited by the absence of external calcium, and can rely on other external cations for propagation (Marsh *et al.*, 1987; Dray *et al.*, 1990b). Conversely, capsaicin-induced neuropeptide release, by exocytosis, has shown a marked extracellular calcium-dependence in tissue or organ studies in, for example, isolated guinea-pig and rat bladder where low external calcium concentrations inhibited capsaicin-induced, peptide-mediated bladder contractions (Maggi *et al.*, 1989). In this particular study, the calcium chelator ethylenediaminetetraacetate (EDTA) was essential for complete inhibition of capsaicin's effects, thus demonstrating the ability of this compound to accumulate intracellular calcium in the presence of low external calcium concentrations.

Maggi (1993) has summarised the available evidence indicating the presence of dual cellular mechanisms for the release of sensory transmitters from nerve endings in CSPANs, both involving the influx of external calcium. The first of these mechanisms

(mode A) occurs at high capsaicin concentrations and involves the intracellular accumulation of calcium through the capsaicin-operated non-selective cation channel that is itself sufficient to evoke neuropeptide release by calcium-induced exocytosis. The second mechanism (mode B) occurs at low capsaicin concentrations that do not induce sufficient intracellular calcium accumulation to cause peptide release, but instead propagate a tetrodotoxin (TTX)-sensitive action potential which in turn stimulates calcium influx through VSCCs, and subsequent neuropeptide release. Hence in addition to its TTX-sensitivity, mode B is also sensitive to VSCC blockers such as the ω-conotoxin (CTX). Conversely, mode A is both TTX- and CTX resistant. This hypothesis has been supported by the functional studies of Lou *et al.* (1992) in the perfused guinea-pig lung where the bronchoconstriction induced by neuropeptide release at low capsaicin concentrations was sensitive to both TTX and the N-type VSCC blocker ω-CTX GVIA. Conversely, the effects of high capsaicin concentrations were TTX- and CTX-resistant in these studies.

1.3.5. Desensitisation

The excitatory actions of capsaicin and other vanilloids on sensory neurons are followed by a refractory state of reduced or non-responsiveness, with prolonged or repeated administration, that is loosely termed 'desensitisation' (see Holzer, 1991; Szolcsányi, 1993 and references therein). This 'trademark' ability of capsaicin and related compounds has promoted intense interest in their use as non-steroidal analgesic and anti-inflammatory agents (Craft and Porecca, 1992; Maggi, 1992; Dray, 1992; Dray and Urban, 1996). More recent attention has focused on RTX and related synthetic, low-pungency compounds such as PPAHV, which show markedly improved desensitising properties relative to their excitatory effects (Szallasi *et al.*, 1996; Szallasi and Blumberg, 1996; Appendino and Szallasi, 1997).

1.3.5.1. Acute versus long-term desensitisation

The effectiveness of capsaicin-induced desensitisation depends on a number of factors including dose, route and frequency of administration (Holzer, 1991; Craft and Porecca, 1992; Dray, 1992), and is broadly divided into the categories of 'acute' and 'long-term' (chronic) desensitisation. Based on a large number of capsaicin-induced desensitisation studies, Szolcsányi (1993) describes a four-part sequence of events with

capsaicin treatment on sensory neurons: (1) excitation involving release of neuropeptides within seconds; (2) "sensory neuron block" where sensory neurons are unresponsive to capsaicin (i.e., neuropeptides are not released) which lasts for hours to several days; (3) degeneration of some neurons and recovery of function of others over several days to weeks; (4) complete degeneration of affected neurons (Table 1.1). Acute desensitisation to capsaicin and related compounds ('specific' desensitisation) is represented by stage 2 of this model and is generally observed when capsaicin is applied repeatedly (with short intervals) to sensory neurons, or at concentrations that are several-fold higher than the threshold for stimulation (excitation) in a given tissue. Conversely, long-term desensitisation is represented by stages 3 and/or 4 of Szolcsányi's model and is more likely to involve neurotoxic impairment of capsaicin-sensitive neurons, and desensitisation to a range of noxious stimuli ('nonspecific' or 'functional' desensitisation) (Holzer, 1991). This form of desensitisation is also more likely to be irreversible, particularly in the neonate or with high dose (>10 mg/kg) systemic capsaicin pretreatment in the adult animal (Holzer, 1991). In the adult, more pronounced systemic desensitisation is achieved using higher total doses (>50 mg/kg), given in three to four injections to minimise death due to respiratory distress and other acute reflexes induced by the agent (Szolcsányi, 1993). However, although pretreatment can ensue irreversible, selective destruction of some CSPANs, it should be noted from Table 1.1 that other CSPANs will continue to show unchanged sensitivity to vanilloids and other noxious stimuli.

1.3.5.2. Mechanisms of vanilloid-induced desensitisation

The mechanisms underlying desensitisation to capsaicin are poorly understood, but it appears likely that those responsible for acute and long-term desensitisation are both distinct and complex (Holzer, 1991; Szolcsányi, 1993; Szallasi, 1994). Desensitisation to capsaicin has been observed in single neuronal cells (eg. Marsh *et al.*, 1987) and is thought to be related to the intracellular accumulation of cations (particularly calcium), osmotic swelling and ultrastructural changes, such as swelling of

TABLE 1.1. Response stages of capsaicin-sensitive primary afferents to vanilloids (reproduced from Szolcsanyi, 1993).

Stage 1 Excitation

(1) Depolarisation of the peripheral, central terminals or the cell body by increasing cation permeability through a tetrodotoxin insensitive and relatively calcium resistant process.

(2) Local depolarisation initiates regenerative spike potentials.

(3) Calcium-dependent release of substance P and other neuropeptides

(4) Time course: seconds; recovery seconds or minutes

Stage 2 Sensory neuron blocking effect

(1) Desensitisation of the sensory receptors to one or more types of stimuli; partial recovery from depolarisation.

(2) No measurable depletion of substance P in peripheral terminals.

(3) Ultrastructural changes, but no degeneration.

(4) Start: immediately after Stage 1; recovery hours or days.

Stage 3 Long-term selective neurotoxic impairment

(1) Loss of excitability of the sensory receptors.

(2) Inhibition of axonal transport and its antagonism by nerve growth factor.

(3) Depletion of the neuropeptide content.

(4) The cell body is preserved with swollen mitochondria, but the axonal processes might degenerate.

(5) Start: within minutes; duration several weeks and some alterations are irreversible.

Stage 4 Irreversible cell destruction

(1) Calcium accumulation and karyolysis within 20 min in neonatal rats.

(2) All types of C-afferents and some large cells are also affected by neonatal treatment.

(3) Capsaicin-sensitive neurons with unchanged sensitivity are still present

mitochondria, leading to impaired neuron function (Holzer, 1991; Szolcsányi, 1993; Bevan and Docherty, 1993). Capsaicin-induced neurotoxicity has also been demonstrated *in vitro* (Wood *et al.*, 1988; Winter *et al.*, 1990) and is also dependent on the influx of calcium and/or sodium, resulting in the subsequent influx of water,

followed by osmotic swelling, damage and cell lysis (Bevan and Docherty, 1993). Interestingly, a cell death response unrelated to apoptosis has also been observed in non-neuronal cells expressing cloned VR1 receptors after prolonged exposure to high external concentrations of capsaicin (Caterina *et al.*, 1997).

Long-term desensitisation may also involve a reduction in vanilloid receptor density since studies with [^{3}H]-RTX have shown receptor loss in several tissues 24 hours after vanilloid pretreatment (Szallasi and Blumberg, 1992; Szallasi *et al.*, 1995; Farkas-Szallasi *et al.*, 1996) requiring up to two months for near-complete recovery (Szallasi, 1995). Other possible sources to account for long-term desensitisation include the inhibition of sensory axonal transport depriving the somata of essential nerve growth factors (NGF) (Miller *et al.*, 1982b) and the depletion of sensory neuropeptides, such as SP and CGRP, seen after vanilloid pretreatment (Buck and Burks, 1986; Holzer, 1988). However, none of the above are likely to be the sole cause of acute or long-term vanilloid-induced desensitisation.

1.4. Non-selective effects of capsaicin

The sensory neuron-selective actions of capsaicin described above are characterised by their occurrence at low effective concentrations of capsaicin and RTX, mediation by specific vanilloid receptor stimulation, and desensitisation with prolonged or repeated stimulation. Conversely, there are 'non-selective' effects of capsaicin that do not exhibit desensitisation, are not mimicked by other vanilloids, occur only at high effective concentrations, and are believed to result from interaction of these compounds with the plasma membrane (i.e. not vanilloid receptor-mediated) (reviewed by Holzer, 1991; Szallasi 1994). Some of the known non-selective responses to capsaicin involve effects on non-mammalian neurons, such as the inhibition of potassium channels in the Ranvier ganglia of the frog (Dubois, 1982) and sodium channels in the giant axon of the crayfish (Yamanaka *et al.*, 1984). In mammals, non-selective actions of capsaicin include effects on ion channels other than the capsaicin-operated channel (reviewed by Holzer, 1991), as well as effects on smooth muscle cells such as the inhibition of cardiac muscle (Franco-Cereceda and Lundberg, 1988) and contraction of vascular smooth muscle (Donnerer and Lembeck, 1982; Duckles, 1986; Saito *et al.*, 1988; Edvinsson *et al.*, 1990).

In the perfused rat hindlimb the repeated infusion of capsaicin-like agents does not induce acute desensitisation to the VO_2 and vasoconstrictor responses in this tissue (Eldershaw *et al.*, 1994) indicating that the effects of these agents may be of the non-selective type described above. However, the concentration and frequency of vanilloid infusion in these studies may not have been sufficient to induce acute desensitisation. It remains to be seen whether or not the effects in this tissue are sensitive to other methods of inducing desensitisation, such as systemic capsaicin pretreatment. Indeed, this latter point is addressed in the studies presented later in this thesis.

1.5. Objectives of the Present Study

Although the metabolic and vascular effects of capsaicin and related compounds in the perfused rat hindlimb are well characterised and are likely to be linked, the mechanisms underlying these responses are poorly understood and have not been related to the mechanisms of capsaicin action in other tissues. The present study attempts to define a role for specific vanilloid receptors, and a sensory efferent function for CSPANs and their neuromodulators, in the responses to capsaicin in the perfused hindlimb preparation. Addressing these points may also determine whether or not the observed effects of vanilloids in this preparation are likely to be non-selective (section *1.4.*). Thus the aims of the study were:

1. To determine the characteristics of vanilloid recognition site(s) which mediate the actions of capsaicin in the perfused rat hindlimb model by the use of known vanilloid antagonists.

2. To investigate the possibility that the biphasic VO_2 effect of vanilloids is due to cooperativity of receptor-ligand binding (by use of the non-cooperative ligand 12-phenylacetate 13-acetate 20-homovanillate); or by separate cellular mechanisms described by others (by co-infusion of tetrodotoxin with capsaicin).

3. To examine the effects of CSPAN ablation by systemic capsaicin pretreatment on the acute metabolic and vascular responses to capsaicin in perfused muscle.

4. To determine the role of sensory neuropeptides in the metabolic and vascular responses to capsaicin in the perfused rat hindlimb by:

   a) studying the effect of post-junctional blockade of neuropeptide receptors, using specific and competitive tachykinin (NK1 and NK2 receptor) and CGRP antagonists, on the acute effects of capsaicin in the perfused rat hindlimb.

   b) Determining the effects of infused SP, NKA, and CGRP in the unstimulated (fully dilated) and NOR-stimulated (vascular preconstricted) perfused rat hindlimb.

Chapter 2

Evidence for Peripheral Vanilloid Receptor Subtypes (VN_1/VN_2) in the Perfused Rat Hindlimb by the use of Competitive and Non-Competitive Capsaicin Antagonists.

2.1 Introduction

Three major developments in the field of vanilloid (capsaicin) pharmacology have had a significant impact on our understanding of their novel receptors (see section *1.3.1* and subsections therein). The first of these was the discovery of capsazepine, an antagonist that is believed to compete directly and selectively for vanilloid receptors (Bevan *et al.*, 1991, 1992; Dickenson and Dray, 1991; Dray *et al.*, 1991; Urban and Dray, 1991). Prior to the discovery of this compound, ruthenium red, an inorganic dye that blocks the capsaicin receptor-cation channel coupling mechanism (Dray *et al.*, 1990a), was the only available capsaicin antagonist and its selectivity at higher concentrations was questionable (Amann and Maggi, 1991).

As reported in section *1.3.1.3*, the ability of capsazepine to act as a selective and competitive vanilloid antagonist has been well documented. Not only has this antagonist helped to confirm the presence of specific vanilloid binding proteins, it has also added support to the notion of vanilloid receptor subtypes since its affinity for vanilloid receptors shows intraspecies variation (Szallasi, 1994). Table 2.1 summarises the dissociation constant estimates for capsazepine at vanilloid receptors obtained from functional and radioligand binding studies in preparations from central nervous system (CNS) and peripheral tissues of the rat. It is evident that the affinity of capsazepine is greatest in peripheral tissues, such as the airways and colon, while binding in the urinary bladder and CNS shows low micromolar affinity. There is an apparent discrepancy in the estimated affinity of capsazepine for inhibiting capsaicin- and RTX-induced Ca^{2+} uptake by cultured DRG neurons (Bevan *et al.*, 1992) compared to that for blocking [^{3}H]-RTX binding to DRG membranes (Szallasi *et al.*, 1993c). However, the latter authors point out that their studies were carried out on adult DRG membrane

preparations, while Bevan and his co-workers used cultured DRG neurons from neonates, where a greater proportion of DRG neurons are sensitive to capsaicin (Lawson and Harper, 1984).

TABLE 2.1. Affinity of capsazepine for vanilloid receptors in central nervous system and peripheral tissues of the rat.

Tissue	Assay	Dissociation constant (K_D or K_i) (μM)	Reference
Dorsal root ganglia	1	3.50	Szallasi *et al.*, 1993c
	3	0.22	Bevan *et al.*, 1992
	1	3.92	Acs *et al.*, 1994b
	1	3.90	Acs *et al.*, 1996
Spinal cord	1	4.0	Szallasi *et al.*, 1993c
	1	3.55	Acs and Blumberg, 1994a
	2	1.30	Szallasi *et al.*, 1995
	1	3.31	Acs *et al.*, 1994b
Urinary bladder	1	5.0	Szallasi *et al.*, 1993c
	1	4.76	Acs *et al.*, 1994b
Airways	1	0.12	Szallasi *et al.*, 1993c
Sciatic nerve	1	3.43	Acs *et al.*, 1994b
Colon	1	0.10	Goso *et al.*, 1993b

Assays: 1, inhibition of [^{3}H]-RTX binding (homogenate radioligand binding); 2, inhibition of [^{3}H]-RTX binding (autoradiography); 3, inhibition of RTX-induced Ca^{2+} uptake

Based on the affinity and binding behaviour (i.e. cooperative vs. non-cooperative) of [^{3}H]-RTX (section *1.3.2.1.*), and the affinity of capsazepine for vanilloid receptors from various tissues (Table 2.1), Szallasi *et al.* (1993c) have hypothesised that there may be at least three vanilloid receptor subtypes in the rat: 1) a CNS-type receptor that binds [^{3}H]-RTX with high affinity in a cooperative manner, but binds capsazepine with micromolar affinity; 2) a urinary bladder-type that binds [^{3}H]-RTX with high affinity and non-cooperatively, and also binds capsazepine with relatively low affinity; and 3) an

airway-type receptor that binds [^{3}H]-RTX with low affinity in a non-cooperative manner, but binds capsazepine with high (nanomolar) affinity.

A recent study postulated the presence of two different vanilloid receptor types (designated VN_1 and VN_2) in rat hindlimb tissue based on both functional and metabolic evidence (Colquhoun *et al.*, 1995). Putative VN_1 and VN_2 receptors were distinguished on the basis of their differing affinities for capsaicin, their different calcium and oxygen dependencies for mediating vasoconstriction, and their ability to stimulate or inhibit VO_2. The present study examines the effect of capsazepine on VO_2 and perfusion pressure concentration-response curves to capsaicin in the perfused rat hindlimb. The rationale behind the use of capsazepine in these studies was two-fold: firstly, the ability of capsazepine to competitively inhibit the actions of capsaicin in muscle would add strength to evidence supporting the presence of specific vanilloid receptors in this tissue; secondly, capsazepine may be able to distinguish between putative hindlimb vanilloid receptor subtypes (VN_1 and VN_2) given the variability of dissociation constant (K_D) estimates for this antagonist in different vanilloid receptor assays (Table 2.1). In addition, the ability of the non-competitive blocker ruthenium red to act as a capsaicin antagonist was examined and compared to the actions of capsazepine in this preparation. Although non-competitive, ruthenium red is thought to selectively impair the vanilloid receptor-channel coupling mechanism by a rapid blocking and unblocking of the stimulated cation channel (Dray *et al.*, 1990a) at low concentrations (10^{-7} M - 10^{-6} M) (Amann and Maggi, 1991). These antagonists proved to be valuable in the present study in determining the nature of the biphasic VO_2 responses produced by vanilloids in the perfused rat hindlimb.

In this chapter, the actions of the novel synthetic vanilloid analogue 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) were also assessed in the hindlimb preparation and compared to capsaicin. In recent radioligand binding studies, PPAHV was shown to bind to central (DRG neuron) vanilloid receptors in a non-cooperative manner (Szallasi *et al.*, 1996). Hence concentration-response curves for PPAHV were constructed in the perfused rat hindlimb and compared to those for capsaicin to determine the likely nature of binding (cooperative vs. non-cooperative) by natural vanilloid analogues in this preparation. The effects of PPAHV were also examined in the presence of a high concentration of capsazepine (10 μM) in order to provide evidence of vanilloid receptor binding by this agent.

It is also conceivable that the dual mechanisms of capsaicin in rat muscle are not the result of the stimulation of the separate receptor subtypes, but rather the separate post-receptor mechanisms described by Maggi (1993), one being TTX-sensitive (low capsaicin concentrations) while the other is TTX-resistant (high capsaicin concentrations) (see section *1.3.4.*). Hence, in the present study, TTX was co-infused with low and high concentrations of capsaicin in an attempt to distinguish between these dual modes of action should they operate in rat muscle.

2.2. Methods

All experimental procedures used were approved by the University of Tasmania Animal Ethics Committee under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Australian Government Press, 1990). Perfusions were conducted using male, 180-200g hooded Wistar rats fed a commercial rat chow diet containing 21.4% protein, 4.6% lipid, 68% carbohydrate and 6% crude fibre with added vitamins and minerals (Gibson's, Hobart). Animals were housed in groups of 6-8 at 21±1°C under a 12h:12h light/dark cycle with an accessible and plentiful water supply.

2.2.1 Rat hindlimb perfusion surgery and procedures

Anaesthesia, surgery and perfusion procedures were performed as described previously (Ruderman *et al.*, 1971; Colquhoun *et al.*, 1988). The animals were anaesthetised with pentobarbitone sodium (60 $mg \cdot kg^{-1}$ i.p.) and ligatures tied at the base of the tail and the left tarsus of the perfused leg. A lateral incision was made at the pubic region followed by an incision along the midline of the abdomen extending to the sternum. The posterior and anterior epigastric vessels, as well as vessels supplying the skin, were ligated and excess body-wall and skin tissue were removed with minimal bleeding. The testes, seminal vesicles, colon and duodenum were subsequently ligated and removed. Evisceration enabled access and ligation of the internal spermatic, iliolumbar and right common iliac vessels, as well as the ureters, to isolate perfusate flow to the left hindquarter. The abdominal aorta and vena cava were carefully separated prior to the injection of heparin (2000 $IU \cdot kg^{-1}$ body weight) into the latter vessel. The vena cava was tied posterior to the branch of the renal veins and the vessel

was cannulated using a 16G surflo catheter (Terumo, USA), as was the aorta using a similar 20G catheter. A final ligature was placed around the abdomen (approximately

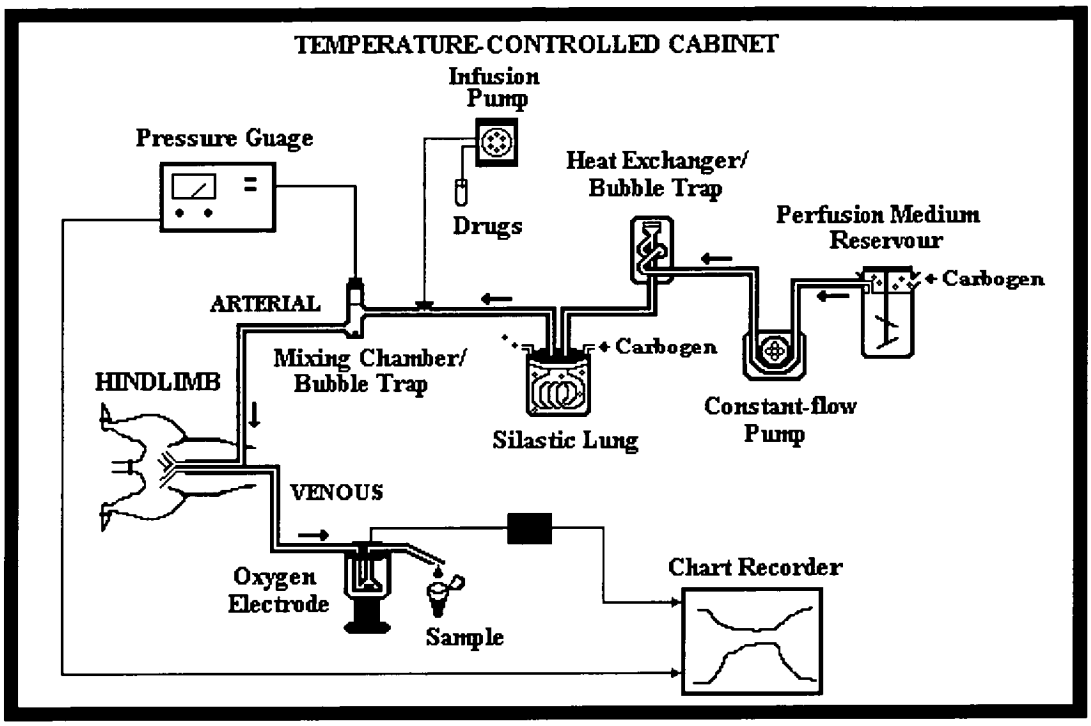


Fig. 2.1. Constant flow perfused rat hindlimb apparatus

L_3 - L_4 vertebrae region) to prevent access of perfusate to the muscles of the back. Following the commencement of perfusate flow, the animal was given a lethal intracardiac injection of pentobarbitone sodium.

Perfusion was performed at 25°C, in a temperature-controlled cabinet (Fig. 2.1), with an erythrocyte-free, Krebs-Ringer bicarbonate buffer containing 8.3 mM glucose, 1.27 mM $CaCl_2$ and 2% w/v dialysed bovine serum albumin (Fraction V). The perfusate reservoir was gassed with 95% O_2-5% CO_2 at 4°C and pumped by a peristaltic pump (Masterflex, Cole Palmer, USA) at a constant flow rate (4.0±0.1 $ml{\cdot}min^{-1}$; 0.27 $ml.min^{-1}{\cdot}g\ muscle^{-1}$) through a heat exchange coil, maintained at 25°C, and a sealed silastic lung continually gassed with the O_2/CO_2 mixture. This ensured constant perfusate temperature and arterial PO_2 levels. The oxygen tension of the venous effluent was measured continuously using an in-line Clark-type oxygen electrode (0.5 ml capacity), maintained at a constant temperature (25°C) by a water jacket, where the activity of oxygen at the electrode is proportional to perfusate PO_2. Perfusion (arterial)

pressure was monitored continuously at a bubble trap proximal to the arterial cannula using a gas-filled pressure transducer.

In perfusions involving sympathetic nerve stimulation, the lumbar sympathetic trunk was ligated, cut and gently pulled away from the aorta in order to make contact with two stainless steel electrodes connected to a stimulator. Liquid paraffin and Parafilm® were placed under the electrodes to prevent stimulation of surrounding tissues. The hindlimb was perfused with the above medium also containing 1 μM tubocurarine to prevent motor neuron activity (Hall *et al.*, 1997). After equilibration, the sympathetic trunk was stimulated electrically (Nerve-Muscle Ştimulator, Hugo Sachs, Germany) using 5V square waves of 5 ms pulse width with variable frequency (0.5 and 5.0 Hz).

2.2.2 Calculation of oxygen uptake

The method of calculation of oxygen consumption (VO_2) has been described previously (Colquhoun *et al.*, 1988). Values for VO_2 calculation and perfusion pressure were taken only after steady state conditions were obtained. Prior to and after each experiment, the oxygen electrode was calibrated with pure oxygen (CIG gases, Australia) and air (O_2 = ~22%). Arterial PO_2 (PaO_2) was determined by connecting the arterial cannula directly to the oxygen electrode, bypassing the perfused tissue. The VO_2 of the perfused tissue was calculated from the difference between PaO_2 and venous PO_2 (PvO_2), the flow rate, and the perfused muscle mass using the following equation:

$$VO_2\ (\mu mol.g^{-1}.h^{-1}) = [1.508 \times (PaO_2 - PvO_2) \times \text{flow rate}]/[\text{perfused muscle mass}]$$

where 1.508 ($\mu mol{\cdot}L^{-1}.mm\ Hg^{-1}$) is the Bunsen coefficient for the solubility of oxygen in human plasma at 25°C (Christoforides *et al.*, 1969). PaO_2 and PvO_2 are in mm Hg; flow rate is in $L.hr^{-1}$; and perfused muscle mass is in g (assumed to be 1/12 of total body mass in a 180-200 g rat; Ruderman *et al.*, 1971).

2.2.3. Materials

Bovine serum albumin (Fraction V) was purchased from Boehringer Mannheim (Australia); pentobarbitone sodium (Nembutal, 60 mg/ml) from Bomac Laboratories Pty. Ltd. (Australia); heparin sodium from David Bull Laboratories (Australia); NaCl,

KCl, KH_2PO_4, $MgSO_4$, $NaHCO_3$, $CaCl_2$ and D-Glucose from Ajax Chemicals Ltd. (Australia).

Capsaicin, noradrenalin (NOR), angiotensin II (AII), ruthenium red, 5-hydroxytryptamine (serotonin or 5-HT), and tetrodotoxin were purchased from the Sigma Chemical Co. (USA); capsazepine was purchased from Research Biochemicals International (USA). Phorbol 12-phenylacetate 13-acetate 12-homovanillate (PPAHV) was a generous gift from Drs. Giovanni Appendino (University of Torino, Italy) and Arpad Szallasi (Washington University, St Louis, USA). Capsazepine was dissolved in ethanol (70-80%), as was capsaicin and PPAHV (50%). All other agents were dissolved in glass-distilled water or normal saline. Where ethanol was used as the vehicle, special care was taken to limit the infusion rate (usually to below 10 µl/min) to avoid any undesired effects that may be produced by this organic solvent.

2.2.4. Agent infusion

The infusion of various agents into the hindlimb commenced only after steady state VO_2 and perfusion pressure had been reached. All agents were either freshly prepared before each experiment, or prepared and then stored at 4°C if chemically stable. Due to the lipophilic nature of vanilloids and their affinity for silicon-based tubing, capsaicin was infused using a syringe pump (Model 2620, Harvard apparatus, USA) driving a 1.0 ml glass syringe (SGE, Australia) equipped with teflon tubing. All other agents were infused with a second pump (Model 355, Sage instruments, Orion Research Inc., USA) using an identical 1.0 ml glass syringe and teflon tubing.

For all experiments, the concentration-response curves for capsaicin or PPAHV were constructed in a cumulative manner with at least two low (nanomolar) and two high (micromolar) concentrations. Each concentration was infused only after VO_2 and perfusion pressure had reached steady state with the preceding concentration. Where the effects of capsaicin were examined against capsazepine, ruthenium red, or TTX, the relevant inhibitor was first infused alone to detect any changes that these drugs might elicit. Increasing concentrations of capsaicin were subsequently co-infused. After the removal of the final capsaicin concentration, the inhibitor was also removed and the preparation was allowed to recover to steady state basal values of VO_2 and perfusion pressure.

In a separate set of control perfusions, capsazepine and ruthenium red were tested against the VO_2 and perfusion pressure changes induced by serotonin (0.35 μM), low (50 nM) and high (5 μM) concentrations of NOR, and AII (3 nM) to establish selectivity of the antagonists for the effects of capsaicin. TTX (0.3 μM) was also infused during the VO_2 and perfusion pressure changes induced by low (0.5 Hz) and high (5 Hz) frequency sympathetic nerve stimulation using the above mentioned protocol.

2.2.5. Data analysis

Statistical analysis of the data was performed by use of Student's *t*-test or by one-way analysis of variance (ANOVA), where applicable. All values are given as the mean ± standard error (s.e.) mean. The Schild plots were constructed from VO_2 and perfusion pressure log concentration-response curves. Dose ratios were estimated at responses between 15% and 85% of the maximum response to capsaicin. The antagonist dissociation constant (K_B) of capsazepine was estimated from the intercept of the Schild regression and abscissa where the intercept = $-\log K_B$ (Kenakin, 1993).

2.3. Results

2.3.1. Capsazepine

For experiments with capsazepine, the mean basal arterial PO_2 was 695.7±5.1 mm Hg and the unstimulated mean venous PO_2 was 366.9±10.3 mm Hg (n=31) corresponding to a mean basal oxygen consumption (VO_2) of 7.9±0.3 μmol. $g.^{-1}$ h^{-1}. Mean arterial perfusion pressure was 28.5±0.7 mm Hg (n=31).

Capsaicin produced a concentration-dependent biphasic response in VO_2 (Fig. 2.2A) and an associated vasoconstriction-induced rise in perfusion pressure (Fig. 2.2B) similar to that reported by Colquhoun *et al.* (1995). A maximum increase in VO_2 (1.2±0.1 μmol. $g.^{-1}$ h^{-1}) was observed at 0.5 μM capsaicin coupled with an increase in perfusion pressure of 9.0±0.9 mm Hg. The highest concentration of capsaicin used (5 μM) produced a strong inhibition of VO_2 (1.9±0.2 μmol. $g.^{-1}$ h^{-1} below basal) with a greater increase in perfusion pressure of 31.6±2.1 mm Hg above basal.

The infusion of capsazepine alone at the concentrations used to inhibit responses to capsaicin failed to alter any basal parameters, and is therefore unlikely to be intrinsically active. Capsazepine did not cause statistically significant alterations

($P>0.05$) in VO_2 and perfusion pressure changes induced by serotonin (5-HT), angiotensin II (AII), or low and high concentrations of noradrenalin (NOR) (Table 2.2).

TABLE 2.2. Changes in VO_2 (ΔVO_2) and perfusion pressure induced by serotonin (5-HT), angiotensin II (AII), and low and high concentrations of noradrenalin (NOR), in the absence and presence of 32 μM capsazepine (CPZ) in the perfused rat hindlimb.

Agents	ΔVO_2 ($\mu mol.\ g.^{-1}\ h^{-1}$)	ΔPerfusion pressure (mmHg)
3nM AII	3.43±0.55	14.0±3.5
3nM AII + 32μM CPZ	3.60±0.25	16.3±4.3
0.35μM 5-HT	-2.35±0.45	70.5±7. 5
0.35μM 5-HT + 32μM CPZ	-2.33±0.43	69.5±4.7
50nM NOR	3.47±0.32	18.3±2.4
50nM NOR + 32μM CPZ	3.50±0.35	19.3±2.9
5μM NOR	-0.83±0.19	181.0±8.6
5μM NOR + 32μM CPZ	-0.93±0.15	175.7±11.1

Values are mean ± s.e. of 3-4 perfusions. Negative ΔVO_2 values occur where agents cause a decrease in VO_2 from basal.

Capsazepine (0.32-32 μM) produced a concentration-dependent rightward shift in the concentration response curves to capsaicin, most evident in the perfusion pressure changes (Fig. 2.2B) and inhibition of VO_2 (Fig. 2.2A). The slopes of Schild plots constructed from dose ratios estimated at different levels of VO_2 inhibition show that as the response becomes greater (i.e. greater inhibition of VO_2) the Schild regressions approach a slope close to unity (Fig. 2.3A).

With the infusion of 3.2 μM and 10 μM capsazepine, the maximum perfusion pressure response was achieved with 5 μM of the agonist (capsaicin). The concentration producing maximum perfusion pressure change increased to 20 μM capsaicin at the highest dose of capsazepine (32 μM). Schild regressions constructed from the perfusion pressure data (Fig. 2.3B) showed a similar pattern to those for VO_2 in that a decrease in

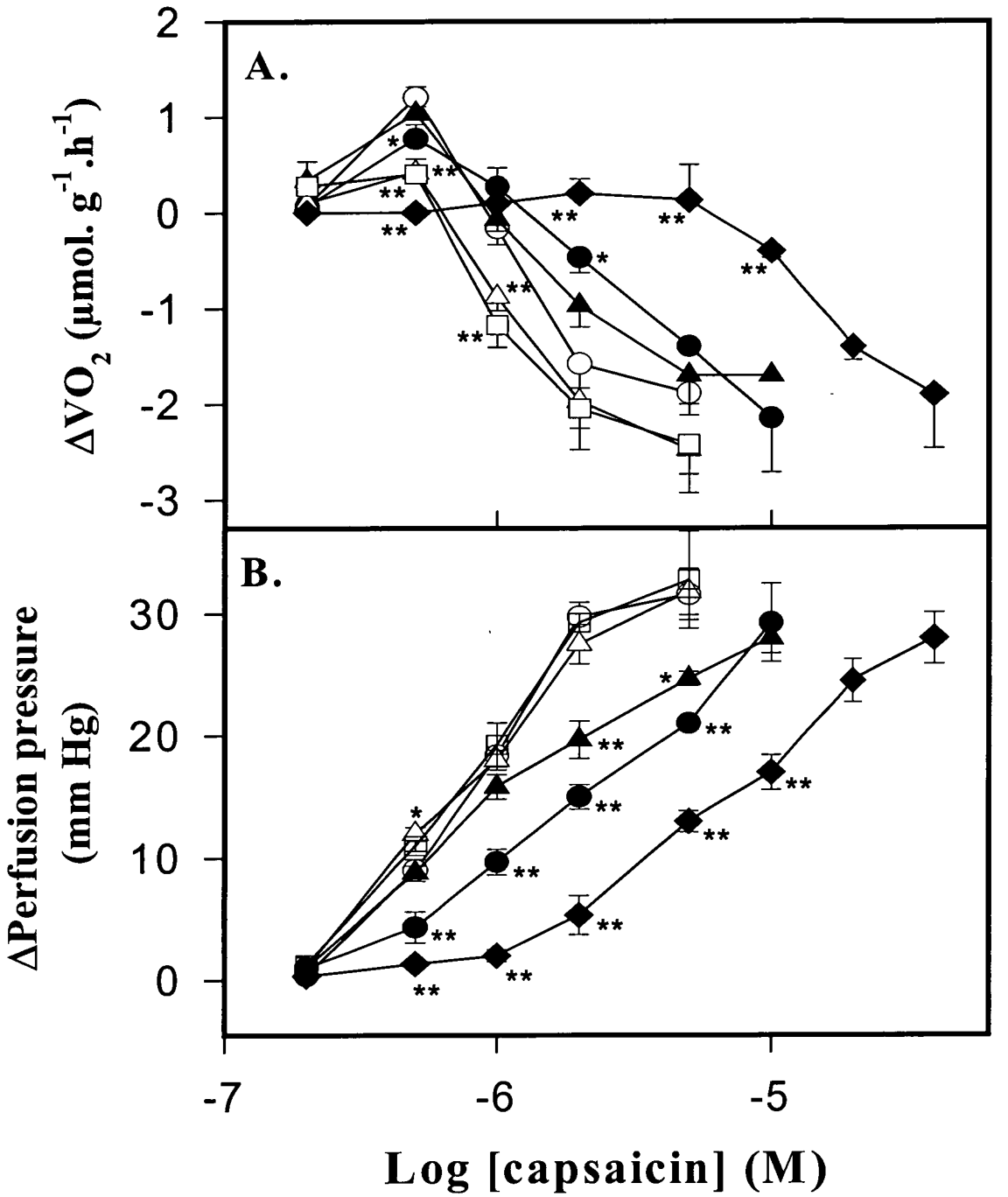


Fig. 2.2 Effect of capsazepine (CPZ) on concentration-response curves for capsaicin-induced changes in (A) oxygen consumption (ΔVO_2), and (B) perfusion pressure in the isolated perfused rat hindlimb: control (○); 0.32 µM CPZ (□); 1.0 µM CPZ (Δ); 3.2 µM CPZ (▲); 10.0 µM CPZ (●); and 32.0 µM CPZ (◆) in 4-11 experiments. All values are mean ± s.e. mean, *P<0.05 **P<0.01 (ANOVA) against control.

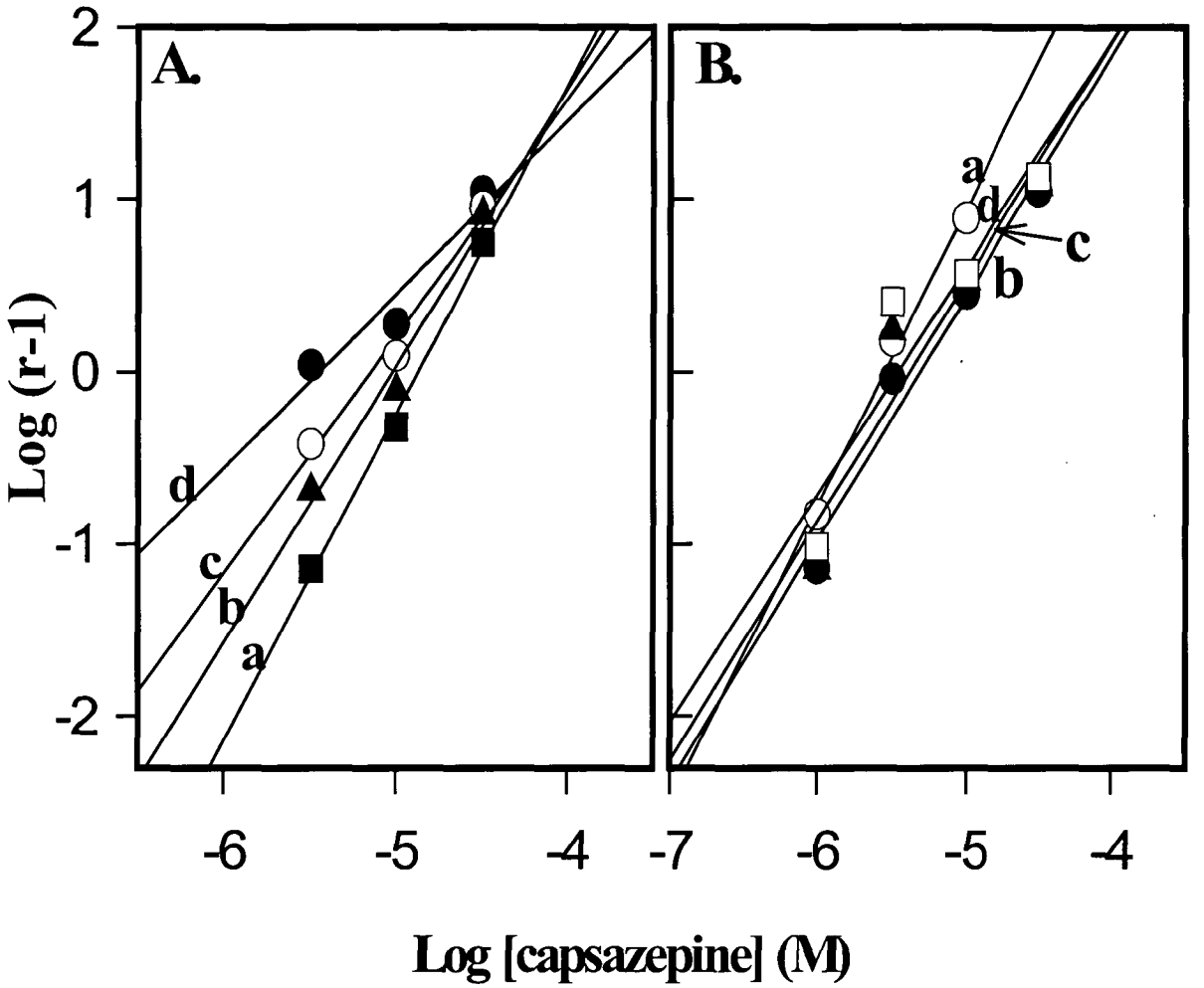


Fig. 2.3 Multiple Schild plots of the data presented in Fig. 2.2. (A) Regressions were constructed from dose ratios estimated at four different levels of the inhibitory VO_2 response: a. 0 μmol. g^{-1}. h^{-1}, slope = 1.87; b. -0.5 μmol. g^{-1}. h^{-1}, slope = 1.61; c. -1.0 μmol. g^{-1}. h^{-1}, slope = 1.37; and d. -1.5 μmol. g^{-1}. h^{-1}, slope = 1.01. The mean K_B derived from the four Schild plots was 8.44 ± 2.08 μM. (B) Regressions constructed from dose ratios estimated at four perfusion pressure responses: a. 15 mm Hg, slope = 2.04; b. 20 mm Hg, slope = 1.52, c. 23 mm Hg, slope = 1.49; and d. 25 mm Hg, slope = 1.45. Mean K_B from the four regressions was 7.28 ± 0.78 μM. Reduction in the number of plot points occurs where some concentration-response curves were shifted to the left of the control curves.

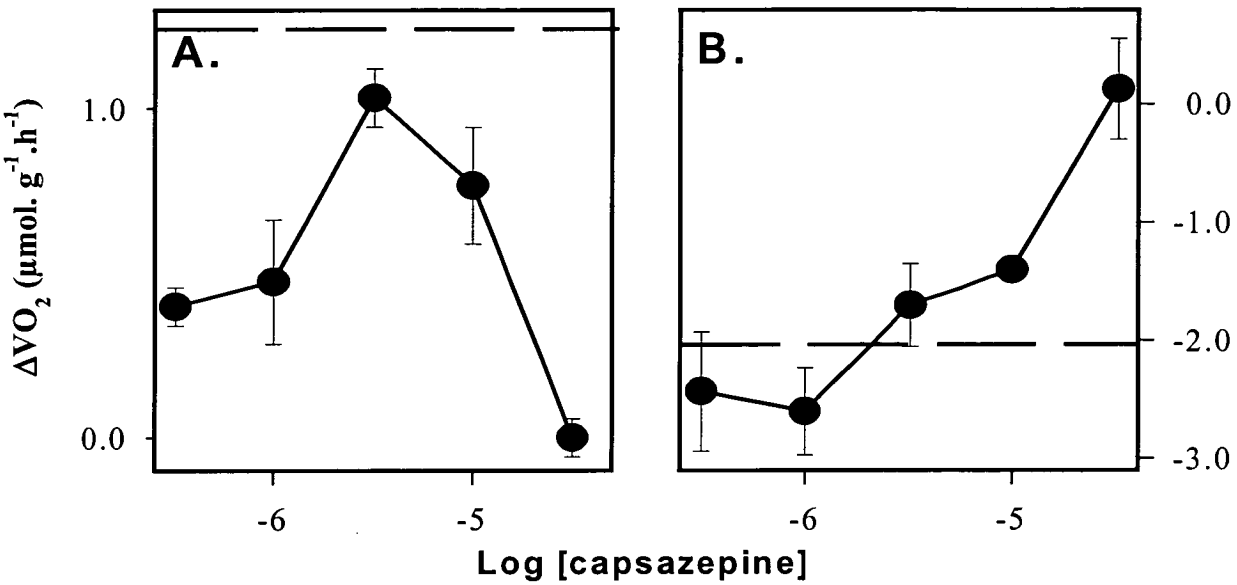


Fig. 2.4. ΔVO_2 response to 5 x 10^{-7} M (panel A) and 5 x 10^{-6} M (panel B) capsaicin with increasing concentrations of CPZ. The dotted line shows the control ΔVO_2 at these concentrations of capsaicin (n = 4-11).

slope is evident from dose ratios estimated at greater responses to capsaicin, although they differ in the range of their slopes and the points of intercept on the abscissa. Thus for Fig. 2.3A the slopes decreased progressively from 1.81 to 1.01, whereas the slopes from Fig. 2.3B decreased from 2.04 to 1.45.

In contrast to capsaicin-induced changes in perfusion pressure, the VO_2 responses show aberrant behaviour in the presence of increasing concentrations of capsazepine (Fig 2.4). At 0.5 μM capsaicin, low concentrations of capsazepine cause an initial fall in response (decreased stimulation of VO_2), followed by a temporary increase in response at higher concentrations of the antagonist (Fig 2.4A). A further increase in capsazepine concentration caused a second but steady decrease in response. At higher concentrations of capsaicin (2-5 μM), the VO_2 response is initially enhanced by low concentrations of capsazepine (greater inhibition of VO_2) followed by a steady decrease in response at higher concentrations of the antagonist (Fig. 2.4B). It appears, therefore, that low concentrations of capsazepine are selectively blocking the stimulatory VO_2 effects produced by low concentrations of capsaicin, resulting in the enhancement of the inhibitory VO_2 effects produced by high concentrations of the vanilloid.

Near maximal inhibition of VO_2 was obtained at high concentrations of the agonist with all concentrations of capsazepine. Maximal low dose responses to capsaicin (stimulation of VO_2) were not achieved at capsazepine concentrations of 1, 10 or 32 μM, an observation likely to be a function of the capsaicin concentrations chosen in the initial infusion protocol.

2.3.2. Ruthenium red

Infusion of ruthenium red (0.01-1.0 μM) alone did not affect mean basal arterial PO_2 (702.6±7.9 mm Hg), venous PO_2 (419.3±4.6 mm Hg), VO_2 (6.8±0.3 μmol. $g.^{-1}$ $h.^{-1}$), or perfusion pressure (24.8±0.3 mm Hg, n=4). The antagonist also failed to show statistically significant inhibition ($P \geq 0.05$) of the VO_2 and perfusion pressure changes induced by 5-HT, AII, and low and high concentrations of NOR (Table 2.3).

TABLE 2.3. Changes in oxygen consumption (ΔVO_2) and perfusion pressure induced by serotonin (5-HT), angiotensin II (AII), and low and high concentrations of noradrenalin (NOR) in the presence and absence of 1 μM ruthenium red (RR).

	ΔVO_2 (μmol. $g.^{-1}$ h^{-1})	**ΔPerfusion pressure (mmHg)**
3nM AII	3.30±0.06	16.0±2.0
3nM AII + 1μM RR	3.40±0.10	16.3±2.2
0.35μM 5-HT	-3.0±0.80	99.0±8.5
0.35μM 5-HT + 1μM RR	-2.75±0.55	109.0±17.0
50nM NOR	3.45±0.26	19.5±2.8
50nM NOR + 1μM RR	3.40±0.25	19.5±2.9
5μM NOR	-0.85±0.17	186.8±6.3
5μM NOR + 1μM RR	-0.78±0.11	183.0±6.1

Values are means±S.E. in 3-4 perfusions. Negative ΔVO_2 values occur where agents cause a decrease in VO_2 from basal.

Ruthenium red produced a concentration-dependent inhibition of both VO_2 (Fig. 2.5A) and perfusion pressure (Fig. 2.5B) responses induced by capsaicin. For both VO_2 and perfusion pressure, the maximal response to the vanilloid agonist was markedly

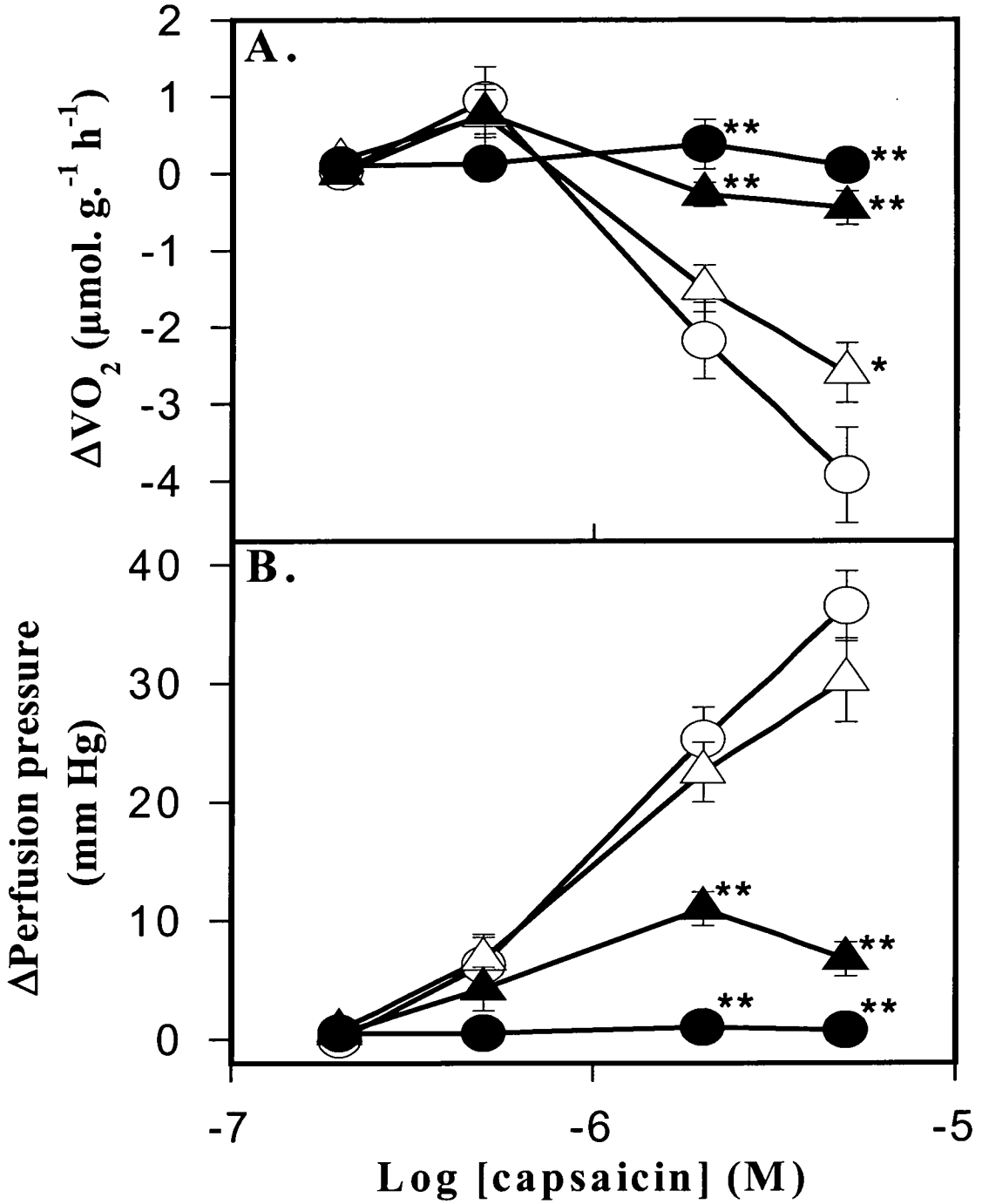


Fig 2.5 Effect of ruthenium red on concentration-response curves for capsaicin-induced changes in (A) oxygen consumption (ΔVO_2); and (B) perfusion pressure in the isolated perfused rat hindlimb: control (○); 0.01 μM ruthenium red (RR) (Δ); 0.1 μM RR (▲); and 1.0 μM RR (●) (all n=4). Values are mean ± s.e. mean. *P≤0.05 **P≤0.01 (ANOVA) against control.

depressed in comparison to the control curve, and was almost completely abolished by the infusion of 1 μM ruthenium red. The stimulation of VO_2 at 0.5 μM capsaicin was inhibited by only 18% with the infusion of 0.1 μM ruthenium red. The same concentration of the dye attenuated the inhibition of VO_2 produced by both 2 μM and 5 μM capsaicin by 87% and 88% respectively.

A similar response was evident for perfusion pressure changes to the infusion of 0.5 μM and 5 μM capsaicin in the presence of 0.1 μM ruthenium red. The increase in pressure produced by the lower dose of capsaicin was inhibited by 32%, while that for the higher doses was reduced by 82%, again indicating selectivity for the blockade of responses to high capsaicin concentrations.

2.3.3. Phorbol 12-phenylacetate 13-acetate 12-homovanillate (PPAHV)

Figure 2.6 shows concentration-response curves for the effect of the synthetic vanilloid analogue PPAHV on VO_2 (panel A) and perfusion pressure (panel B) in the perfused rat hindlimb. PPAHV induced a biphasic effect on VO_2, in association with concentration-dependent vasoconstriction, that is characteristic of the infusion of other natural vanilloids into this preparation. In the presence of capsazepine (10 μM), concentration-response curves for PPAHV-induced changes in VO_2 and perfusion pressure showed a parallel shift to the right (Fig. 2.6) similar to that seen for capsaicin in the presence of the antagonist (Fig. 2.2). The observed maximum stimulation of VO_2 was obtained by the infusion of 0.2 μM PPAHV (ΔVO_2, 0.83 ± 0.06 μmol $g.^{-1}$ h^{-1}) and was accompanied by a mild increase in PP (ΔPP, 8.0 ± 1.1 mm Hg). This stimulation of VO_2 was significantly ($P<0.05$) inhibited by capsazepine (ΔVO_2, -0.10 ± 0.04 μmol $g.^{-1}$ h^{-1}), and there was no apparent perfusion pressure response. However, a similar observed maximum stimulation of VO_2, to that seen in the control concentration response curve, was evident with the infusion of 0.4 μM PPAHV in the presence of capsazepine (ΔVO_2, 0.80 ± 0.10 μmol $g.^{-1}$ h^{-1}). The highest concentration of PPAHV infused caused inhibition of VO_2 (ΔVO_2, -2.73 ± 0.51 μmol $g.^{-1}$ h^{-1}) and a strong vasoconstrictor effect (ΔPP, 42.0 ± 1.2 mm Hg). The inhibition of VO_2 at this concentration of PPAHV was not significantly ($P>0.05$) inhibited by capsazepine (ΔVO_2, -2.0 ± 0.35 μmol $g.^{-1}$ h^{-1}) suggesting that a depression of the maximum response to the agonist is unlikely. However, the highest observed perfusion pressure response

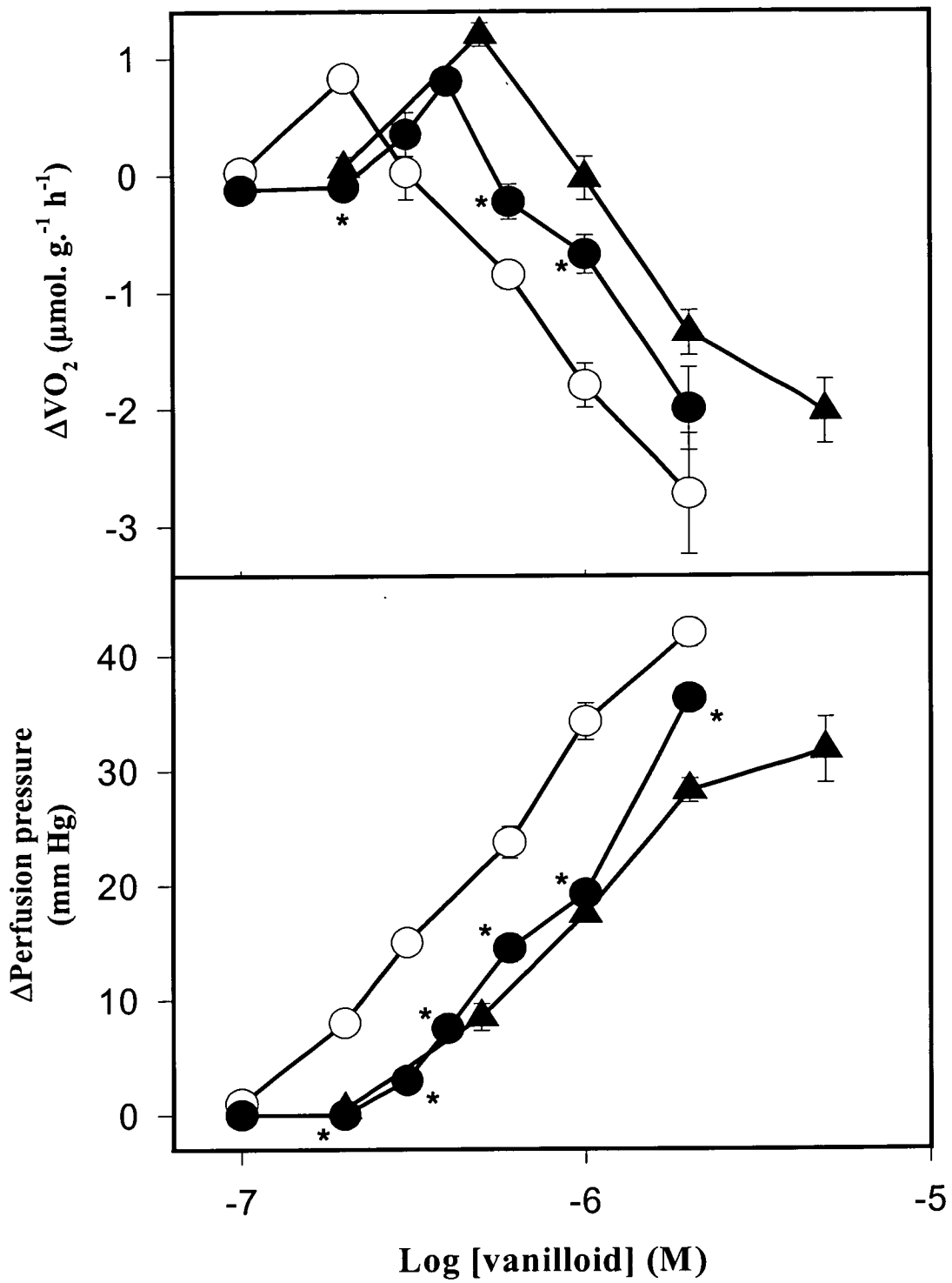


Fig. 2.6. Concentration-response curves for oxygen consumption (panel A) and perfusion pressure (panel B) in the perfused rat hindlimb for 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) alone (○) and in the presence of 10 μM capsazepine (●), compared to data for capsaicin from Fig. 2.2 (▲) (n = 4-5). Values are mean ± s.e. mean. *P<0.05 PPAHV + capsazepine vs. PPAHV control.

was significantly ($P<0.05$) lowered by capsazepine (ΔPP, 36.3 ± 1.1 mm Hg), although this could possibly be overcome by increasing the concentration of PPAHV.

Comparison of the VO_2 and perfusion pressure concentration response curves for PPAHV and those for capsaicin from Fig. 2.2 (Fig. 2.6) reveals that PPAHV is slightly more potent than the natural analogue. This could not be confirmed by estimating the half-maximal effective concentration (EC_{50}) for these ligands as maximum responses for PPAHV-induced perfusion pressure change and inhibition of VO_2 were not observed at the concentrations used.

2.3.4. Tetrodotoxin (TTX)

The data presented in figure 2.7 shows that TTX (0.3μM) failed to inhibit the VO_2 and perfusion pressure responses to both low (nanomolar) and high (micromolar) concentrations of capsaicin. In fact, in the presence of TTX, there was a statistically significant increase in ΔVO_2 from the control curve (without TTX) that may reflect some sensitisation of the tissue during repeated infusion of capsaicin.

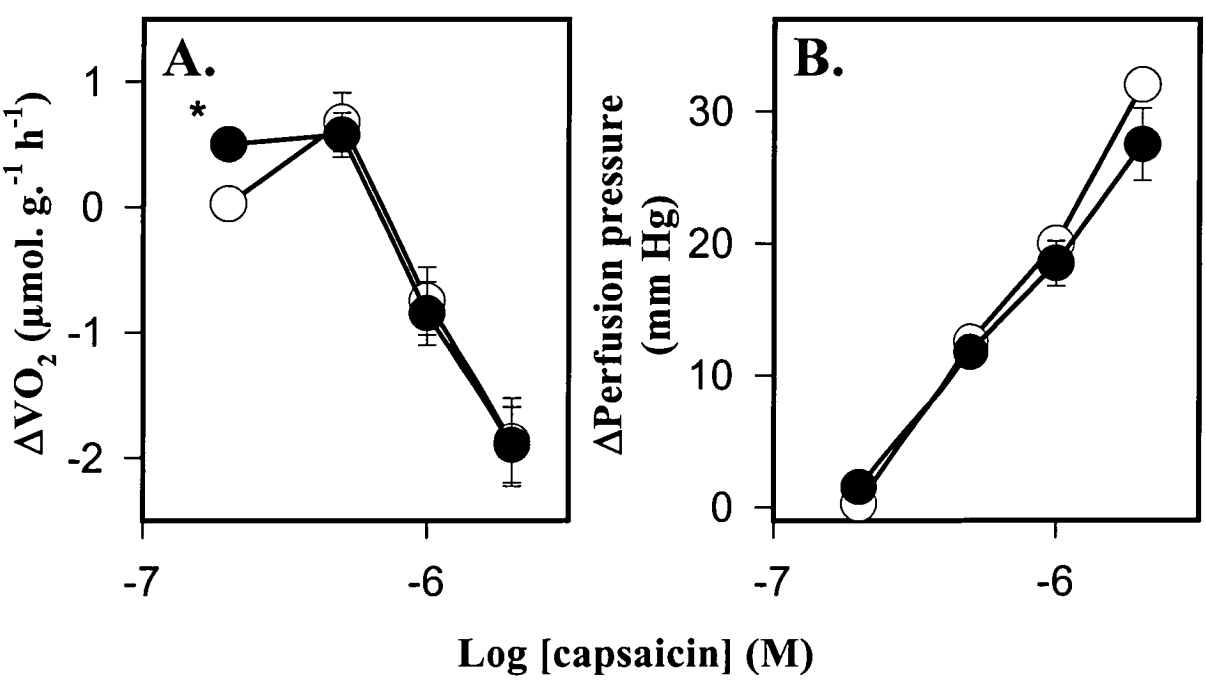


Fig. 2.7 Concentration-response curves for oxygen consumption (panel A) and perfusion pressure (panel B) for capsaicin in the perfused rat hindlimb alone (○), or in the presence of 0.3 μM tetrodotoxin(●) (n = 4). *P<0.05.

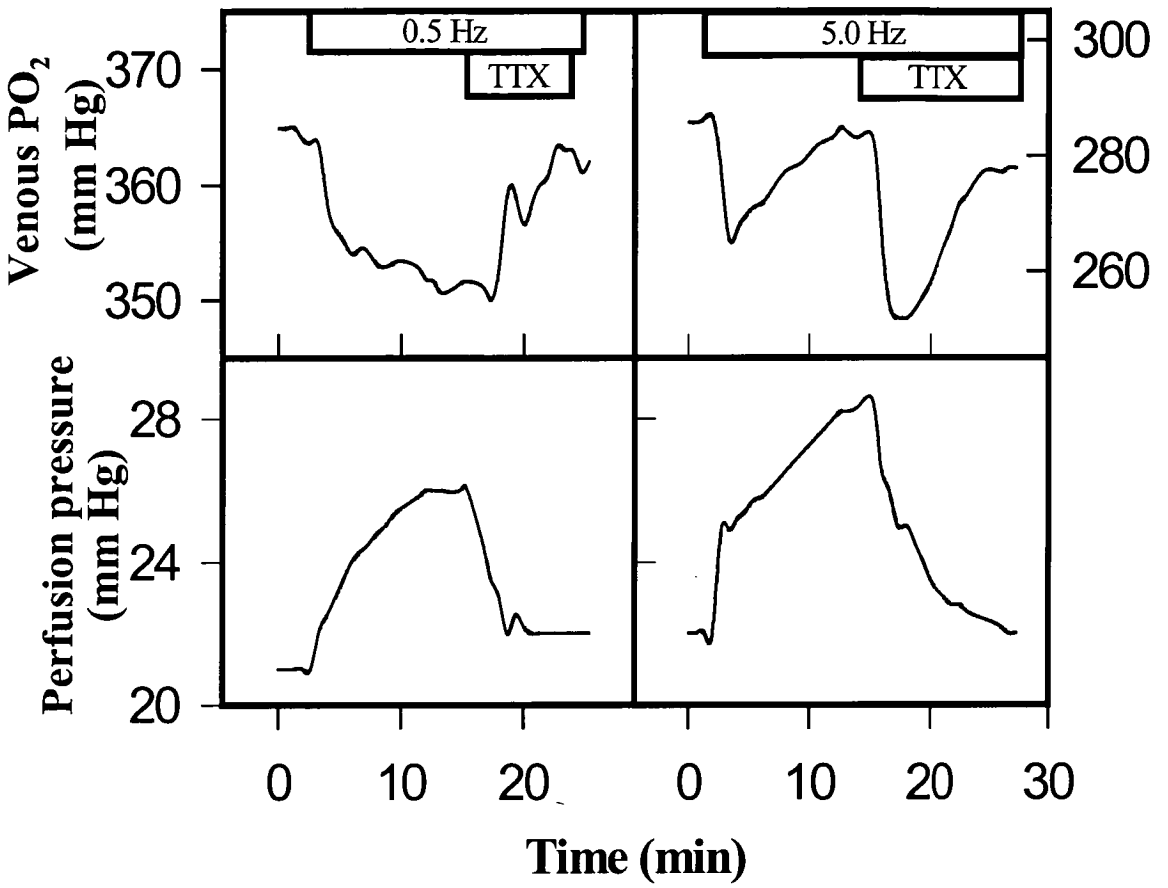


Fig. 2.8. Inhibition by tetrodotoxin (TTX) (0.3 μM) of venous PO_2 and perfusion pressure changes induced by low (0.5 Hz) and high (5 Hz) sympathetic nerve stimulation. Repeat experiments with TTX produced very similar results.

Conversely, the same concentration of TTX almost completely blocked the vasoconstriction (increased perfusion pressure) induced by low and high frequency sympathetic nerve stimulation, and altered the concomitant changes in venous PO_2 in the rat hindlimb preparation (Fig. 2.8).

2.4. Discussion

2.4.1 Vanilloid receptor heterogeneity in a skeletal muscle preparation

The biphasic nature of the capsaicin-induced muscle VO_2 responses alone suggests the operation of a dual vanilloid receptor mechanism, stimulated by a single agonist, where a higher affinity receptor may be responsible for the initial stimulation of

VO_2, and a lower affinity receptor is possibly responsible for the inhibition of VO_2. The argument in favour of this vanilloid receptor subtype hypothesis has been strengthened by recent studies examining the effect of submicromolar and micromolar concentrations of capsaicin under various conditions of metabolic challenge in the hindlimb preparation (Colquhoun *et al.*, 1995). The ability of capsaicin to vasoconstrict and to stimulate (low concentrations) or inhibit (high concentrations) VO_2 was assessed for its dependence on external calcium; and its dependence on oxygen under conditions of hypoxia, and during disruption of cell mitochondria by cyanide and azide. The main findings to emerge from these studies are summarised in Table 2.4.

Table 2.4. Proposed classification criteria for VN_1 and VN_2 receptors in perfused rat muscle (reproduced from Colquhoun *et al.*, 1995, with permission from the authors).

Receptor type	VN_1	VN_2
Oxygen consumption	increased	decreased
Vasoconstriction	strong	moderate
Affinity for vanilloid	high	low
Dependent on external Ca^{2+}	yes	no[1]
Dependent on O_2	yes	no
Lactate production (steady state)	increased	decreased[2]

[1]Independent of $[Ca^{2+}]$ but may require some Ca^{2+} for full agonist effect as inhibition of VO_2 is less than that in the presence of Ca^{2+}.

[2]After removal of capsaicin there is a "wash-out" peak of lactate

These results clearly indicate that the actions of low and high concentrations of capsaicin result from the activation of two separate mechanisms and, conceivably, these different actions of capsaicin may be under the control of at least two receptor subtypes (putative VN_1 and VN_2 receptors) in muscle.

The findings presented in this chapter, demonstrating the effect of infusing capsaicin into the perfused rat hindlimb, are in good agreement with those published

previously (Cameron-Smith *et al.*, 1990; Colquhoun *et al.*, 1995). By employing competitive and non-competitive vanilloid antagonists, the present data has added strength to the notion that capsaicin-mediated responses in perfused rat muscle are mediated by capsaicin binding to specific vanilloid recognition sites, and that there may be at least two receptor subtypes (VN_1 and VN_2) controlling the observed actions of the vanilloid.

Infusion of capsazepine into the rat hindlimb produced competitive antagonism of capsaicin-stimulated changes in VO_2 and perfusion pressure (Figs. 2.2A and 2.2B). The inability of capsazepine to inhibit the actions of other non-vanilloid agonists, that produce effects similar to those induced by vanilloids, suggests that capsazepine is highly specific for the actions of capsaicin. Similar patterns of specific competitive antagonism by capsazepine have been observed in other systems where the effects of capsaicin are believed to be receptor-mediated (Bevan *et al.*, 1992; Maggi *et al.*, 1993a) and, therefore, it is likely that capsaicin also binds to distinct vanilloid recognition sites in rat hindlimb tissue.

Conceptually, the effects of overlapping and opposing receptor subtypes has been considered as an explanation for bell-shaped concentration-response curves (Rovati and Nicosia, 1994). These authors have attempted to distinguish the bell-shaped concentration-response curves produced by opposing receptor subtypes from those produced by partial agonists. A similar model developed earlier by Szabadi (1977) proposed that an antagonist acting preferentially on one of the opposing receptor types will potentiate the effect produced by the other, and that the net effect will be evident in the observed concentration-response curve.

Data presented in this chapter have demonstrated similar effects by selectively inhibiting the stimulatory (low dose/VN_1) component of the biphasic VO_2 response to capsaicin with 0.32 μM and 1 μM capsazepine. This resulted in potentiation of the inhibitory (high dose/VN_2) VO_2 component. That is, a single agonist (capsaicin) seems to act on two receptor types, one stimulatory and one inhibitory, to produce an overall biphasic curve, as demonstrated in Fig. 2.9. The data suggests that the VN_1 receptor is responsible for the increases in VO_2 seen at submicromolar concentrations of capsaicin. The lower affinity VN_2 receptor produces a more pronounced inhibitory effect on VO_2 at capsaicin concentrations exceeding 1 μM. The theoretical curve VN_1+VN_2 represents the combined effect of the two receptors and closely resembles the biphasic

curve derived from the infusion of increasing concentrations of capsaicin. It is proposed that low concentrations of capsazepine (0.32 μM and 1 μM) have selectively blocked the putative VN_1 receptor and, consequently, the resultant stimulation of VO_2.

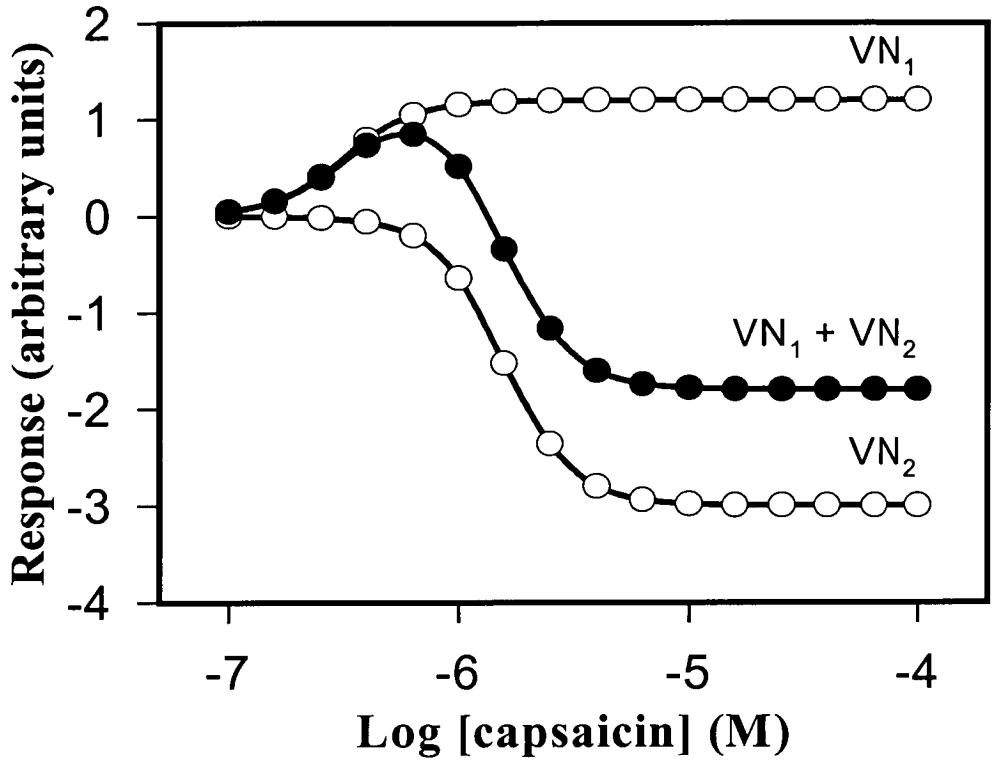


Fig. 2.9 A concentration-response curve model of oxygen consumption for putative vanilloid subtypes VN_1 and VN_2 in the perfused rat hindlimb. VN_1 and VN_2 curves were constructed by applying the four parameter logistic equation used by Rovati and Nicosia (1994). EC_{50} (0.3 μM) and IC_{50} (1.6 μM) values for this equation were estimated from Fig.2.2. Maximal VN_1 and VN_2 responses were estimated at 1.2 and -3.0 units, respectively. These receptor types show different affinities for capsaicin that is reflected in the observed concentration-response curve, represented by the sum of the theoretical VN_1 and VN_2 curves (VN_1+ VN_2). The inhibition of the VN_1 receptor type by low concentrations of capsazepine shifts the balance of agonist occupancy in favour of the opposing VN_2 receptor, hence causing the effect produced by this latter receptor type to predominate before it is also inhibited at higher capsazepine concentrations.

Hence a shift in the balance of agonist occupancy has occurred in favour of the putative VN_2 (inhibitory) receptor. As a result, the inhibitory VO_2 response has become more pronounced in the overall concentration-response curves produced in the presence of low concentrations of capsazepine.

The complex interaction of agonist and antagonist interacting with two opposing receptor types, having different affinities, may have altered the dose ratios sufficiently to account for the steepness of some of the Schild regression slopes. It is conceivable that as the inhibition of VO_2 becomes greater (by increasing the capsaicin concentration) the emphasis of binding is placed in favour of the putative VN_2 receptor. As a result, the Schild plots derived at higher inhibitory VO_2 responses (having slopes close to unity) resemble more closely a single receptor-agonist/antagonist interaction. Steepness in Schild plots can also represent a non-equilibrium steady state between the antagonist and receptor, or the presence of a saturable antagonist removal mechanism (Kenakin, 1993). In this study, an antagonist removal mechanism may be present due to the use of bovine serum albumin (BSA) medium to act as the necessary oncotic agent. In perfusions where 2% BSA is replaced by 4% Ficoll® (Pharmacia, Sweden) in the perfusion medium, capsaicin shows a 10-20 fold greater potency for inducing changes in VO_2 and vascular tension (C.D. Griffiths, Honours Thesis, Division of Biochemistry, University of Tasmania, 1994). Capsazepine, being a structural analog of capsaicin, may also have a similar susceptibility to binding to BSA. However, on this basis alone it is difficult to account for the decreases in Schild plot slope when the dose ratios were estimated at greater agonist responses.

By contrast, Schild regressions obtained for the perfusion pressure data do not fully support a dual receptor proposal (Fig 2.3B). These observations may be a result of the inability to distinguish between the similar vasoconstrictor responses produced by putative VN_1 and VN_2 receptors, unlike the opposing responses on VO_2 where VN_1 and VN_2 effects can be distinguished with the infusion of low concentrations of capsazepine.

In the hindlimb preparation, 1 μM ruthenium red produced a specific but non-competitive inhibition of capsaicin-induced responses (Fig. 2.5) similar to that seen in the rat *vas deferens* and urinary bladder where the maximal responses to the vanilloid are potently antagonised (Maggi *et al.*, 1993a). Again, this inhibition has been shown to be specific since the above concentration of ruthenium red used to block the effects

of capsaicin did not alter the actions of other non-vanilloid agonists. The infusion of nanomolar concentrations of the dye produced strong inhibition of the VO_2 and perfusion pressure effects of high (micromolar) concentrations of capsaicin, while low dose capsaicin responses showed only mild inhibition. The apparent selective antagonism of high dose capsaicin responses by low concentrations of ruthenium red further supports the notion of a dual vanilloid receptor system. Given the suggested intimacy between putative vanilloid receptors and non-selective cation channels (James *et al.*, 1993), it follows that if different vanilloid receptor types exist, then there could also be heterogeneity in the receptor-channel coupling system as a whole. The pattern of inhibition by low dose (nanomolar) ruthenium red in rat hindlimb tissue certainly suggests that this may be the case.

2.4.2. Cooperative versus non-cooperative vanilloid receptor binding

In rat DRG membrane preparations, increasing concentrations of unlabelled RTX produces a biphasic effect on [^{3}H]-RTX binding (an initial increase followed by inhibition) suggesting cooperativity of ligand binding to the vanilloid receptor (Szallasi *et al.*, 1993b, Acs and Blumberg, 1994b). Hence, the binding of [^{3}H]-RTX is initially enhanced by low concentrations of unlabelled ligand, followed by concentration-dependent inhibition of radioligand binding at higher RTX concentrations. However, when non-radioactive RTX is substituted with increasing concentrations of PPAHV, only monophasic inhibition of [^{3}H]-RTX binding is evident (Szallasi *et al.*, 1996). Consequently, the binding behaviour of this ligand to CNS vanilloid receptors is thought to be non-cooperative in nature.

The shape of the biphasic competition curves from CNS radioligand binding studies bears a striking resemblance to the biphasic nature of VO_2 concentration-response curves for capsaicin in the perfused rat hindlimb (Colquhoun *et al.*, 1995; section *1.2.3.*), suggesting that vanilloids may bind to hindlimb receptors in a cooperative fashion. Hence if PPAHV were to produce monophasic VO_2 concentration-response curves in rat muscle, then this may strengthen the above hypothesis. However, data presented in this chapter using increasing concentrations of PPAHV showed that this ligand induced a biphasic effect on muscle VO_2, with vasoconstriction, similar to that seen with the infusion of increasing concentrations of capsaicin. The apparent affinity of this synthetic analogue, relative to the affinity of capsaicin and RTX

(Eldershaw *et al.*, 1994), in this preparation is in excellent agreement with those determined in radioligand binding assays (Szallasi *et al.*, 1996). Furthermore, it is very likely that PPAHV is inducing its effects by binding to specific vanilloid receptors since the vanilloid antagonist capsazepine (10 μM) caused a parallel shift to the right, without diminution of some observed maximum responses, in the concentration-response curves for this agonist (Fig. 2.6). This inhibition of PPAHV by capsazepine was very similar to that observed for capsaicin by the antagonist (Fig. 2.2), suggesting that all three ligands interact with the same vanilloid recognition sites.

2.4.3. Capsaicin cellular mechanisms in muscle

The observations with TTX in the perfused rat hindlimb are in contrast to those found by Lou *et al.* (1992) (see section *1.3.4.*) using the same concentration of the toxin (0.3 μM). The TTX-resistant nature of all capsaicin-mediated changes in the rat hindlimb raises speculation regarding the cellular mechanisms promoted by vanilloids in this preparation. In view of recent reports by Akopian *et al.* (1996) of a TTX-resistant voltage-gated sodium channel on capsaicin-sensitive rat dorsal root ganglion (DRG) neurones, it follows that capsaicin could possibly activate the opening of similar channels in rat skeletal muscle. However, attempts by these authors to detect such a channel in skeletal muscle were unsuccessful. Consequently, there exists the possibility that the cellular mechanisms promoted by capsaicin at low concentrations in the perfused rat hindlimb preparation could differ from other tissues, but more evidence is undoubtedly required.

Thus the mechanisms by which capsaicin induces vascular and biphasic VO_2 changes in the perfused rat hindlimb are likely to involve at least two distinct receptor subtypes. However, no attempt has been made in this chapter to assess the likely post receptor mechanisms employed by capsaicin in this tissue, such as involvement of capsaicin-sensitive neurons and their peptide transmitters. Instead, this forms the basis of the studies presented in Chapter 3.

Chapter 3

The Potential Role for Capsaicin-Sensitive Neurons and Their Peptide Transmitters in the Acute Responses to Capsaicin in Perfused Rat Muscle.

3.1 Introduction

The findings from the previous chapter demonstrated that the dual effects of capsaicin in perfused muscle VO_2 are likely to be mediated by specific vanilloid receptor subtypes. However, the underlying mechanisms by which VN_1 and VN_2 receptors produce these responses are poorly understood. In other tissues, vanilloid receptors are thought to be coupled to non-selective cation channels on certain C-type and Aδ-type sensory neurons (James *et al.*, 1993) and the recent cloning of a capsaicin receptor from DRG has confirmed this long-held belief (Caterina *et al.*, 1997). Stimulation of these ion-channel receptors facilitates the intracellular accumulation of calcium ions and co-release of several neuropeptide transmitters, most notably calcitonin gene-related peptide (CGRP), and the tachykinins substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). These sensory peptides may produce a variety of biological responses, including changes in vascular tone and permeability (reviewed by Holzer, 1998), smooth muscle contraction, and inflammation (reviewed by Holzer, 1991). A hallmark of capsaicin action on peptide-containing sensory neurons is its ability to induce a refractory state of desensitisation with prolonged or repeated *in vitro* application, or following systemic administration (see section *1.3.5.*). Indeed, it is this unique property of capsaicin and its structural analogues that has resulted in considerable interest in the use of these compounds as antinociceptive and anti-inflammatory agents (Bevan and Szolcsanyi, 1990; Dray, 1992; Maggi, 1992; Campbell *et al.*, 1993, Dray and Urban, 1996).

The actions of the tachykinin peptides are mediated by at least three receptor subtypes: SP-preferring NK1, NKA-preferring NK2 and NKB-preferring NK3 receptors (reviewed by Mussap *et al.*, 1993; Maggi *et al*, 1993b; Regoli *et al.*, 1994). All three of

the known tachykinin receptor subtypes have been cloned (Masu *et al.*, 1987; Yokota *et al.*, 1989; Shigemoto *et al.*, 1990; Ingi *et al.*, 1991) and are known to belong to the G-protein-coupled receptor family (Nakanishi *et al.*, 1993). NK1 receptors are widely distributed in both the CNS and peripheral tissues, whereas NK2 receptors are found mainly in peripheral tissues (predominantly on smooth muscle) and NK3 receptors in the CNS, although the latter are expressed in the rat portal vein and guinea-pig myenteric plexus (Mastrangelo *et al.*, 1987; Guard *et al.*, 1990). While each of the tachykinins have preferred receptors, it is widely accepted that the selectivity of each peptide for these receptors is poor and that many of their physiological actions may result from their interaction with more than one tachykinin receptor type (reviewed in Otsuka and Yoshioka, 1993). To date, attempts to synthesize selective agonists, demonstrating high selectivity for each receptor type, have been met with limited success (Mussap *et al.*, 1993; Regoli *et al.*, 1994; McLean and Lowe, 1994). Although some synthetic peptide agonists display high selectivity for their preferred receptors, the lability of these peptides to enzymatic degradation raises concerns about their usefulness for characterising tachykinin receptors in different tissues (McLean and Lowe, 1994). Similarly, peptide antagonists for tachykinin receptors are prone to degradation and, in addition, may induce undesirable effects such as mast-cell degranulation, partial agonism, neurotoxicity and local anaesthetic properties (reviewed in Maggi *et al.*, 1993b). Hence the development of the first non-peptide antagonists for NK1 (Snider *et al.*, 1991; Garret *et al.*, 1991, McLean *et al.*, 1993), NK2 (Emonds-Alt *et al.*, 1992), and more recently NK3 receptors (Emonds-Alt *et al.*, 1994), showing high receptor affinity and high selectivity, has made an important contribution to the study of tachykinin receptor pharmacology in individual tissues.

Receptors for CGRP are tentatively divided into two distinct subtypes ($CGRP_1$ and $CGRP_2$) based on the differing ability of C-terminal fragments of the peptide to antagonise the actions of intact CGRP in different preparations (reviewed by Poyner, 1995). One of these fragments, $CGRP_{(8-37)}$, is the only commercially available CGRP antagonist at present and was first recognised for its ability to competitively inhibit CGRP- and calcitonin-induced cAMP production in a rat liver plasma membrane preparation (Chiba *et al.*, 1989). Shorter C-terminal fragments retain similar selective antagonism, but possess markedly reduced affinity for CGRP receptors when compared to $CGRP_{(8-37)}$ (Bell and McDermott, 1996). Since its discovery, this truncated peptide

has been used in a variety of functional preparations from different species to competitively inhibit the actions of CGRP. The estimated affinity (given as pA_2, the log concentration of antagonist required to produce a dose ratio equal to 2) of $CGRP_{(8-37)}$ for its receptors in several rat tissue preparations is presented in Table 3.1, and demonstrates the ambiguity experienced with the use of this antagonist in different functional assays.

TABLE 3.1 - pA_2 estimates from $CGRP_{(8-37)}$ inhibition of CGRP responses in several rat tissue preparations

Tissue	Assay	pA_2[1]	Reference
Liver membranes	cAMP accumulation	8.35	Chiba *et al*, 1989
Vas deferens	relaxation	6.77	Dennis *et al.*, 1990
L6 myocytes	cAMP accumulation	8.38	Poyner *et al.*, 1992
Vas deferens	relaxation	5.85	Giuliani *et al.*, 1992
Adipocytes	glycoprotein synthesis	6.91	Casini *et al.*, 1991
Mesentery	vasodilatation	8.50	Foulkes *et al.*, 1991

[1]pA_2 is the log concentration of antagonist required to produce a two-fold shift to the right (i.e. dose ratio = 2) in a concentration-response curve for a competing agonist.

The data presented in this chapter attempts to define a role for capsaicin-sensitive neurons, their major peptide transmitters (SP, NKA and CGRP), and neuropeptide receptors in capsaicin-induced responses in the perfused hindlimb by employing the following pharmacological tools and techniques:

- non-peptide and peptide antagonists for tachykinin (NK1 and NK2) and CGRP (putative $CGRP_1$) receptors, respectively
- endogenous peptide ligands for tachykinin (NK1, NK2 and NK3) and CGRP receptors
- systemic capsaicin pretreatment which is known to ablate capsaicin-sensitive primary afferent neurons (CSPANs) (section *1.3.5.*).

Concentration-response curves for capsaicin-induced changes in perfused hindlimb VO_2 and perfusion pressure were constructed in the presence of the competitive non-peptide antagonists CP-99,994 (NK1 receptors) and SR 48968 (NK2 receptors), and the truncated peptide antagonist $CGRP_{(8-37)}$ ($CGRP_1$ receptors). Animals were also pretreated with capsaicin and the perfused hindlimb responses to the vanilloid were recorded 1, 7 and 14 days after pretreatment. In addition, the effects of infusing the neuropeptides SP, NKA, NKB and CGRP were studied in an attempt to mimic the actions of capsaicin and to further characterise the peptides and receptors involved.

3.2 Methods

3.2.1. Rat hindlimb perfusion.

Animals were anaesthetised with pentobarbitone sodium (60 mg/kg) and their left hindlimbs perfused according to the method described previously (section *2.2.1*, Ruderman *et al.*, 1971; Colquhoun *et al.*, 1988).

3.2.2. Materials

Substance P, neurokinin A, neurokinin B, calcitonin gene-related peptide and $CGRP_{(8-37)}$ were purchased from Auspep (Australia); capsaicin, Sigmacote® and phosphoramidon from the Sigma Chemical Company; bovine serum albumin (Fraction V) from Boehringer Mannheim (Australia) and pentobarbitone sodium (Nembutal, 60 mg/ml) from Bomac Laboratories (Australia). Non-peptide tachykinin antagonists were generous gifts: (2S,3S)-3-(2-methoxybenzyl)amino-2-phenylpiperidine (CP-99,994) from Dr. S.B. Kadin, Pfizer Inc., Groton, CT, U.S.A., and (S)-N-methyl-N[4-(4-acetylamino-4-phenyl piperidino)-2-(3,4dichlorophenyl)butyl]benzamide (SR 48968) from Dr. X. Emonds-Alt, Sanofi Recherche, Montpellier, France. All other reagents were of analytical grade.

Neuropeptides were dissolved into 20 µl aliquots using a 0.01 M acetic acid solution containing 1% β-mercaptoethanol and stored at -20°C to maintain chemical stability. The aliquots were then diluted, as needed, with 0.9% NaCl so that the acetate and β-mercaptoethanol concentrations were negligible. Due to the lipophilic nature of capsaicin, it was dissolved in 50% ethanol and care was taken to keep the infusion rates

low (usually below 10 µl/min) to avoid vehicle-induced effects. All other agents were dissolved in normal saline (0.9% w/v).

3.2.3 Agent infusion

Capsaicin and the neuropeptides were infused using a syringe pump (Model 2620, Harvard Apparatus Inc., USA) driving a 1.0 ml glass syringe (SGE) equipped with teflon tubing. Other agents were infused using similar infusion pumps (Model 355, Sage instruments, Orion Research Inc. USA, or Model 11 microinfusion, Harvard Apparatus Inc., USA) also with an identical 1.0 ml glass syringe and teflon tubing. In perfusions using SP, NKA, NKB or CGRP, all glassware was cleaned and silanised with Sigmacote® (prior to infusion) to prevent peptide adhesion to glass surfaces.

In perfusions using either CP-99994, SR 48968 or $CGRP_{(8-37)}$, control concentration-response curves for VO_2 and perfusion pressure were first obtained by the cumulative infusion of increasing concentrations of capsaicin followed by a period of recovery after drug removal. Following re-establishment of basal VO_2 and perfusion pressure, either CP-99994 (0.1, 0.5 or 1 µM), SR 48968 (0.1, 1 or 10 µM) or $CGRP_{(8-37)}$ (1 µM) was infused alone for approximately 5 minutes, and then co-infused whilst the capsaicin concentration-response curve was repeated. When infused alone, none of the antagonists induced detectable changes in either basal VO_2 or perfusion pressure.

The neutral endopeptidase inhibitor phosphoramidon (5 µM) was co-infused with each neuropeptide (following phosphoramidon alone for 5 minutes) to prevent enzymatic degradation. Several concentrations of SP, NKA, NKB or CGRP were infused to enable the construction of concentration-response curves and estimation of half-maximal effective concentration (EC_{50}) for each peptide.

3.2.4. Capsaicin pretreatment

Capsaicin pretreatment was given according to the pre-established method of Cui and Himms-Hagen (1992a; 1992b) with a minor modification to the anaesthetic used. Briefly, a total dose of 125 mg/kg capsaicin was administered, under anaesthesia (40-60 mg/kg pentobarbitone), in four s.c. injections over a three day period (day 1, 12.5 mg/kg; day 2, 2 x 25 mg/kg; day 3, 62.5 mg/kg). Care was taken to limit the dose of pentobarbitone on day two of the protocol where the animals were anaesthetised twice (morning and evening). Injections were given behind the neck or near the rump where

s.c. injection was easier due to the loose skin at these locations. Injections of the vehicle (10% Tween 80, 10% ethanol in normal saline) were given to control animals. Hindlimbs were perfused 1, 7 or 14 days after the final capsaicin (or vehicle) injection and the responses to the infusion of several concentrations of the vanilloid were recorded. The animals were weighed on each day of the injection protocol and prior to perfusion to examine the effect of the pretreatment on overall growth (see Appendix 2).

3.2.5. Data analysis

Statistical analysis was performed using Student's t-test, one-way analysis of variance (ANOVA) or ANOVA on ranks (Kruskal-Wallis analysis), or by one-way repeated measures ANOVA or one-way repeated measures ANOVA on ranks (Friedman analysis), where applicable. All ANOVAs were subjected to Student-Newman-Keuls multiple comparisons where $P<0.05$ was considered statistically significant. The half maximal effective concentration (EC_{50}) and maximum effect (E_{max}) for SP, NKA and NKB were estimated from VO_2 and perfusion pressure concentration-response curves for individual experiments. For NKB, the maximum VO_2 effect was not obtained, hence the EC_{50} for this peptide was estimated using the mean E_{max} from the SP and NKA experiments. In capsaicin pretreatment experiments, the EC_{50} for the acute effects of capsaicin was estimated from individual concentration-response curves for perfusion pressure.

3.3. Results

For all experiments, mean basal hindlimb VO_2 was 8.90 ± 1.13 μmol. $g.^{-1}$ h^{-1} while that for perfusion pressure was 24.93 ± 0.35 mm Hg (n = 65). All drug-induced changes in these parameters were estimated under steady state conditions. At all concentrations tested, CP-99,994, SR 48968 and $CGRP_{(8-37)}$ failed to alter any of the above parameters when infused in the absence of other agents.

3.3.1. CP-99,994

Concentration-response curves for capsaicin were characteristically biphasic for VO_2, as seen previously (Colquhoun *et al.*, 1995; Chapter 2), with a concentration-dependent increase in perfusion pressure that is indicative of vasoconstriction (Fig. 3.1).

Consecutive concentration-response curves for capsaicin were very similar, as indicated by the data obtained using an ineffective concentration of CP-99,994 (Fig. 3.1A, D). The observed maximal stimulation of VO_2 was induced by 0.4 μM capsaicin (ΔVO_2, 1.35±0.14 μmol. $g.^{-1}$ h^{-1} above basal VO_2) followed by inhibition of VO_2 at concentrations above 1 μM with the observed maximal inhibition of VO_2 at 2 μM capsaicin (-2.25±0.35 μmol. $g.^{-1}$ h^{-1} below basal VO_2, Fig 3.1C). The non-peptide NK-1 receptor antagonist, CP-99,994 (1 μM), selectively inhibited some, but not all, of the stimulation of VO_2 induced by capsaicin (ΔVO_2, 0.78 ± 0.06 μmol. $g.^{-1}$ h^{-1}; $P<0.05$, Fig 3.1C).

A lower concentration of CP-99,994 (0.5 μM) had a similar but less pronounced effect on VO_2, ($P<0.05$) (Fig. 3.1B). While some statistically significant ($P<0.05$) differences in the perfusion pressure responses to capsaicin were evident in the presence of CP-99,994, these changes were not consistent over the three antagonist concentrations used (Fig. 3.1D-F).

3.3.2. SR 48968

At a concentration of 1 μM, the selective NK2 receptor antagonist SR 48968 significantly inhibited ($P<0.05$) the maximum stimulation of VO_2 induced by 0.4 μM capsaicin (ΔVO_2, control, 1.06±0.13 μmol. $g.^{-1}$ h^{-1}; SR 48968, 0.52±0.24 μmol. $g.^{-1}$ h^{-1}, Fig. 3.2B). Although the stimulation of VO_2 at a lower concentration of capsaicin (0.25 μM) was potentiated in the presence of 1 μM SR 48968 (Fig 3.2B), this effect is unlikely to be caused by the antagonist since there is occasionally mild sensitisation to the VO_2 stimulatory response when a capsaicin dose-response curve is repeated in the absence of other agents (Griffiths, Eldershaw and Colquhoun, unpublished observations). The infusion of a high concentration of SR 48968 (10 μM) produced further blockade of the maximum capsaicin-induced stimulation of VO_2 (ΔVO_2, control, 1.03±0.08 μmol. $g.^{-1}$ h^{-1}; SR 48968, 0.17±0.30 μmol. $g.^{-1}$ h^{-1},

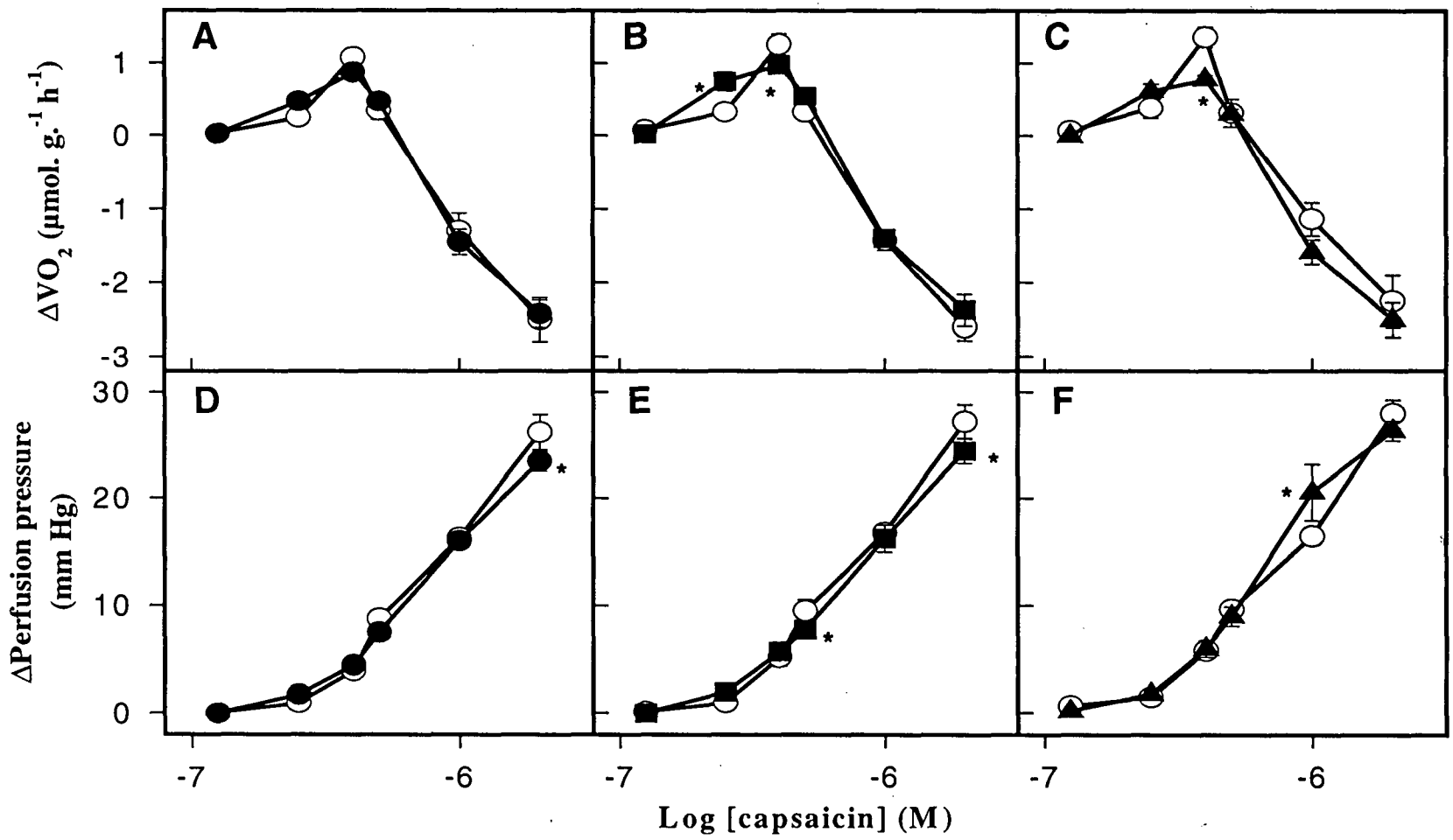


Fig. 3.1 Effect of the NK-1 receptor antagonist CP-99,994 on concentration-response curves for capsaicin-induced changes in oxygen consumption (panels A,B,C); and perfusion pressure (panels D,E,F) in the perfused rat hindlimb. Control (○), 0.1 μM (●), 0.5 μM (■) and 1.0 μM (▲) CP-99,994. *P<0.05 from control (one-way repeated measures ANOVA). Values are mean±s.e. mean in 5-6 experiments.

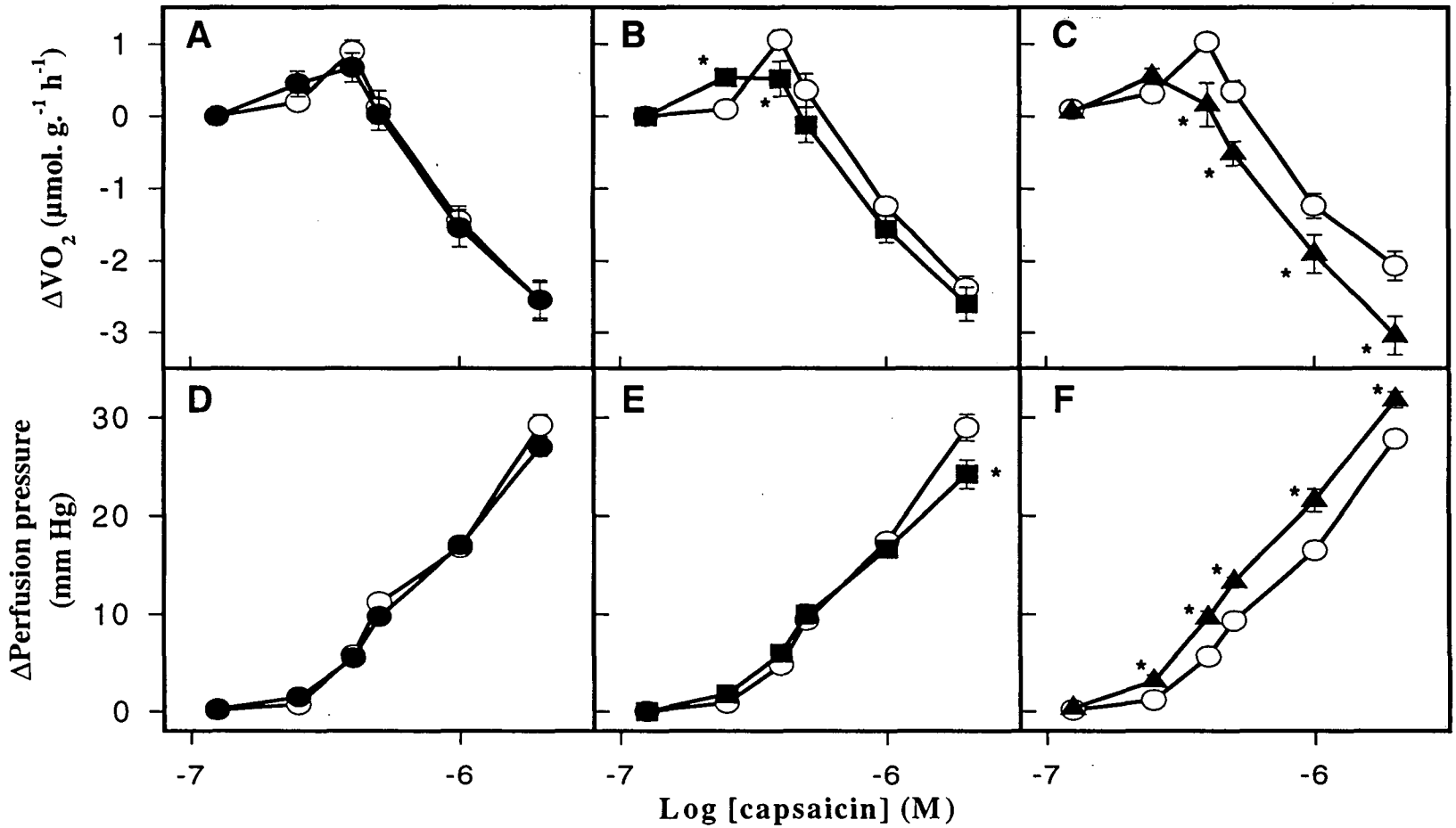


Fig. 3.2. Effect of the NK2 (neurokinin A) receptor antagonist SR 48968 on concentration-response curves for capsaicin-induced changes in oxygen consumption (panels A,B,C); and perfusion pressure (panels D,E,F) in the perfused rat hindlimb. Control (○), 0.1 μM (●), 1.0 μM (■) and 10.0 μM (▲) SR48968. *P<0.05 from control (one-way repeated measures ANOVA). Values are mean±s.e. mean in 5-6 experiments.

P<0.05, Fig 3.2C), while the inhibition of VO_2 produced by a high concentration of the vanilloid (2 μM) was significantly (P<0.05) enhanced (ΔVO_2, control, -2.07±0.20 μmol. $g.^{-1}$ h^{-1}; SR48968, -3.04±0.26 μmol. $g.^{-1}$ h^{-1}). Vasoconstriction at all concentrations of capsaicin was also significantly (P<0.05) enhanced by 10 μM SR 48968 (Fig 3.2F).

3.3.3. $CGRP_{(8-37)}$

Infusion of the CGRP antagonist, $CGRP_{(8-37)}$, significantly (P<0.05) increased the stimulation of VO_2 induced by 0.125 and 0.25 μM capsaicin (ΔVO_2, control, 0.03 ± 0.03 and 0.13±0.06 μmol. $g.^{-1}$ h^{-1}; $CGRP_{(8-37)}$, 0.28 ± 0.09 and 0.80±0.09 μmol. $g.^{-1}$ h^{-1}, respectively) but did not significantly increase the observed maximal stimulation of VO_2 produced by the infusion of 0.4 μM capsaicin (Fig 3.3A). The inhibition of VO_2 induced by 1 μM capsaicin was significantly (P<0.05) enhanced by the co-infusion of $CGRP_{(8-37)}$ (ΔVO_2, control, -1.13±0.29 μmol. $g.^{-1}$ h^{-1}; $CGRP_{(8-37)}$, -2.08±0.15 μmol. $g.^{-1}$ h^{-1}, Fig 3.3A) while vasoconstriction at 1 and 2 μM capsaicin was markedly (P<0.05) increased (ΔPP; control, 16.5±0.7 mm Hg and 29.3±2.0 mm Hg, respectively; $CGRP_{(8-37)}$, 36.8±2.1 mm Hg and 46.0±3.1 mm Hg, respectively, Fig. 3.3B).

3.3.4. Substance P, neurokinin A, neurokinin B and calcitonin gene-related peptide

Infusion of neutral endopeptidase inhibitor phosphoramidon (5 μM) alone had no detectable effect on either basal VO_2 or perfusion pressure. The co-infusion of increasing doses of SP with phosphoramidon produced a concentration-dependent increase in VO_2 (Fig 3.4A and Table 3.2) and induced mild vasoconstriction (Fig 3.4B and Table 3.2). Increasing the dose of SP to micromolar concentrations caused some attenuation of the VO_2 increase, while the effect on perfusion pressure plateaued. NKA, also co-infused with phosphoramidon, produced similar effects on hindlimb VO_2 and perfusion pressure, but was approximately 10-fold more potent than SP (Fig 3.4 and Table 3.2). The infusion of NKB, with phosphoramidon, stimulated a small but reproducible change in VO_2, however, maximum VO_2 was not obtained using concentrations of NKB that induced a maximum change in vascular tone (Fig 3.4 and Table 3.2). At all concentrations tested, CGRP had no detectable effect on perfused hindlimb VO_2 or perfusion pressure (Fig 3.4).

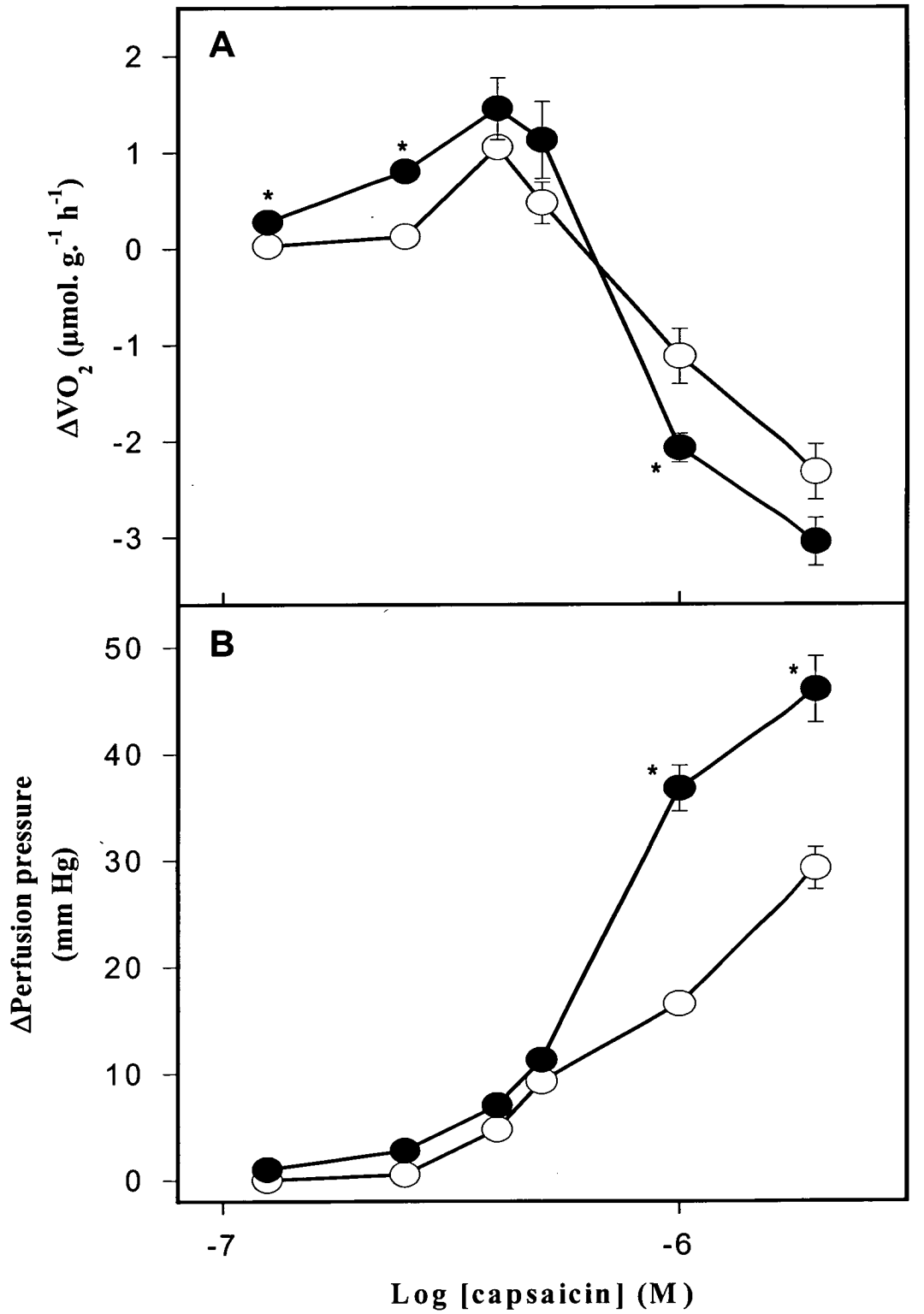


Fig. 3.3. Effect of the calcitonin gene-related peptide (CGRP) receptor antagonist $CGRP_{(8-37)}$ on concentration-response curves for capsaicin-induced changes in oxygen consumption (panel A); and perfusion pressure (panel B) in the perfused rat hindlimb. Control (○) and 1.0 μM $CGRP_{(8-37)}$ (●).*P<0.05 from control (one-way repeated measures ANOVA). Values are mean±s.e. mean in 5-6 experiments.

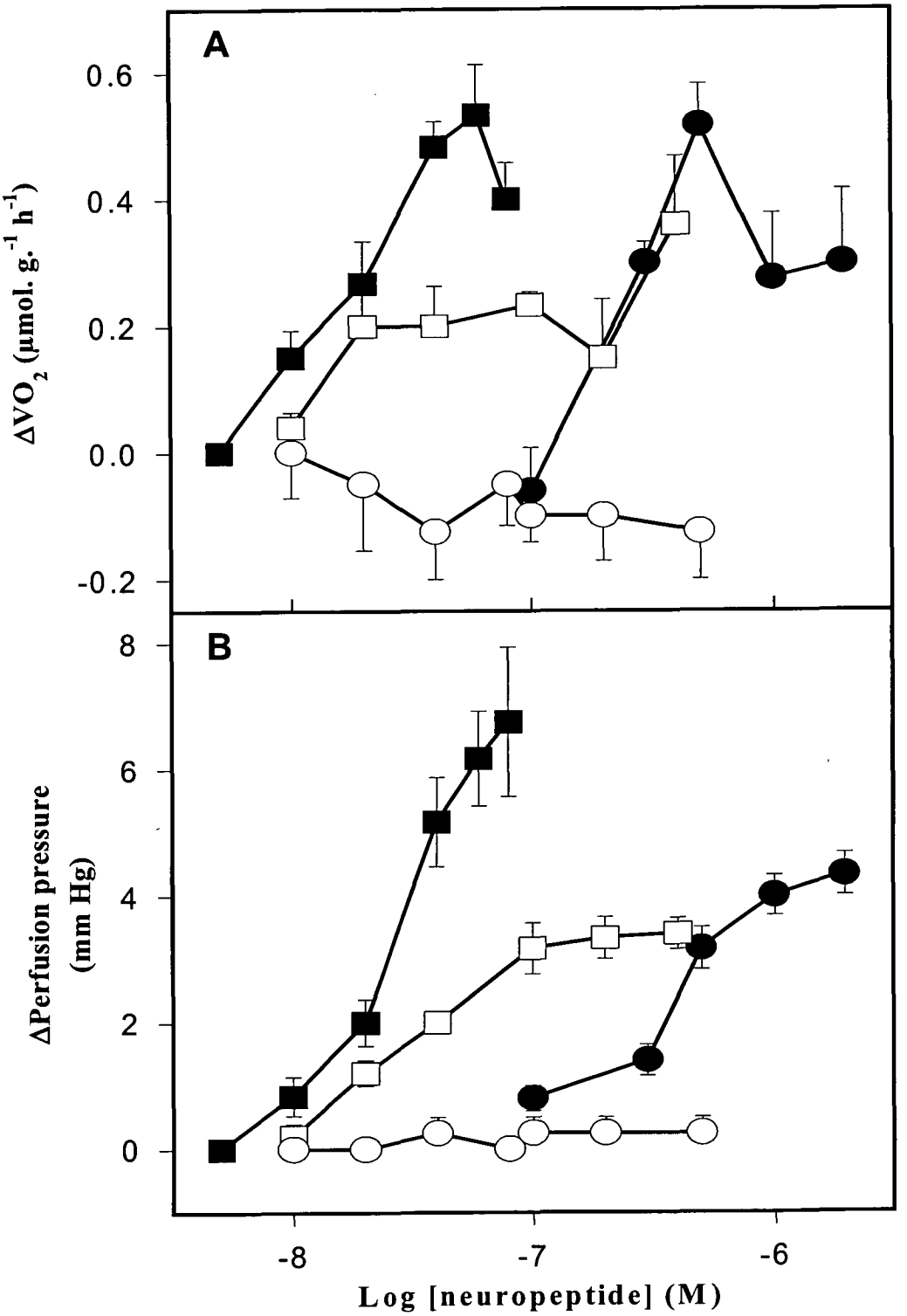


Fig. 3.4. Effect of substance P (SP) (●), neurokinin A (NKA) (■), neurokinin B (NKB) (□), and calcitonin gene-related peptide (CGRP) (○) on oxygen consumption (panel A) and perfusion pressure (panel B) in the perfused rat hindlimb. In all experiments, SP, NKA, NKB and CGRP were co-infused with the neutral endopeptidase inhibitor phosphoramidon (5 μM). Values are mean±s.e. mean in 4-6 experiments.

TABLE 3.2. Maximum change in perfusion pressure (ΔPP) and oxygen consumption (ΔVO_2), and concentration producing 50 percent of maximum response (EC_{50}) for substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) in the perfused rat hindlimb.

		ΔPP		ΔVO_2	
		E_{max}	EC_{50}	E_{max}	EC_{50}
Neuropeptide	**n**	(mm Hg)	(nM)	(μmol. $g.^{-1}$ h^{-1})	(nM)
SP	5	4.33±0.33	352.0±25.70	0.52±0.07	269±23.00
NKA	4	6.75±1.18	25.5±2.72	0.53±0.08	21.2±2.98
NKB	5	3.4±0.25	34.4±5.19	-	71.8±29.20[1]

Values are mean ± s.e. mean

[1]EC_{50} estimated using mean of E_{max} (ΔVO_2) for SP and NKA.

3.3.5. Capsaicin pretreatment

Results of the weight trials during capsaicin and vehicle pretreatment are presented in Appendix 2. Capsaicin pretreated animals showed a statistically significant ($P<0.05$) weight loss on day three of the injection protocol, but only in the groups perfused seven and fourteen days after pretreatment. Animals perfused one day after capsaicin pretreatment did experience some weight loss on day three of the injection protocol, but this did not reach statistical significance ($P>0.05$). This was possibly due to the greater standard error and smaller total sample size of the group. When the weights of all animals were pooled (total group), no statistically significant differences were observed over the three day pretreatment period. The weight losses induced by pretreatment with capsaicin are unlikely to have affected the acute responses to capsaicin in the perfused hindlimb since the mean final weight of all groups were very similar.

Figure 3.5 shows VO_2 and perfusion pressure responses to capsaicin in hindlimbs perfused 1, 7 and 14 days after vehicle or systemic capsaicin pretreatment. Stimulation of VO_2 induced by submicromolar concentrations of capsaicin was inhibited ($P<0.05$) 1 day after capsaicin-pretreatment (maximum ΔVO_2; control, 0.98±0.23 μmol. $g.^{-1}$ h^{-1} ; capsaicin pretreated, 0.08±0.04 μmol. $g.^{-1}$ h^{-1}, Fig 3.5A). However, the increase in

perfusion pressure induced by 2 μM capsaicin was markedly ($P<0.05$) enhanced (ΔPP; control, 23.2±1.4 mm Hg; capsaicin pretreated, 35.8±3.3 mm Hg, Fig 3.5D). Seven and 14 days after capsaicin-pretreatment, the stimulation of VO_2 and vasoconstriction induced by low concentrations of capsaicin was completely restored, whereas the maximum inhibition of VO_2 by 2 μM capsaicin was significantly ($P<0.05$) enhanced compared with vehicle-pretreated controls (ΔVO_2; 7 days, control, -3.18±0.06, capsaicin pretreated, -4.27±0.46; 14 days, control, -3.02±0.25, capsaicin pretreated, -4.52±0.40 μmol. $g.^{-1}$ h^{-1}, Fig 3.5B, C). The maximum vasoconstriction at micromolar concentrations of capsaicin was also greatly increased 7 days after capsaicin pretreatment, and increased further after 14 days (Fig 3.5E, F and Table 3.4). In addition, the half-maximal effective concentration (EC_{50}) for capsaicin, estimated from the perfusion pressure concentration-response curves, was significantly ($P<0.01$) lower in animals perfused 7 and 14 days after capsaicin pretreatment (Table 3.3).

TABLE 3.3. Maximum capsaicin-induced change in perfusion pressure (ΔPP) and capsaicin concentration producing 50 percent of maximum response (EC_{50}) in the perfused rat hindlimb, 1, 7 and 14 days after vehicle- or capsaicin-pretreatment.

Pretreatment	n	Days after pretreatment	E_{max} (Δmm Hg)	EC_{50} (μM)
Vehicle	5	1	30.2 ± 2.22	1.07 ± 0.04
Capsaicin	5	1	35.8 ± 3.28	0.99 ± 0.07
Vehicle	4	7	31.0 ±1.78	1.02 ± 0.05
Capsaicin	4	7	42.3 ± 0.33**	0.74 ± 0.04**
Vehicle	5	14	29.3 ± 1.80	0.96 ± 0.05
Capsaicin	5	14	47.0 ± 4.38**	0.66 ± 0.05**

Values are mean ± s.e. mean. **P<0.01 from corresponding vehicle pretreated controls.

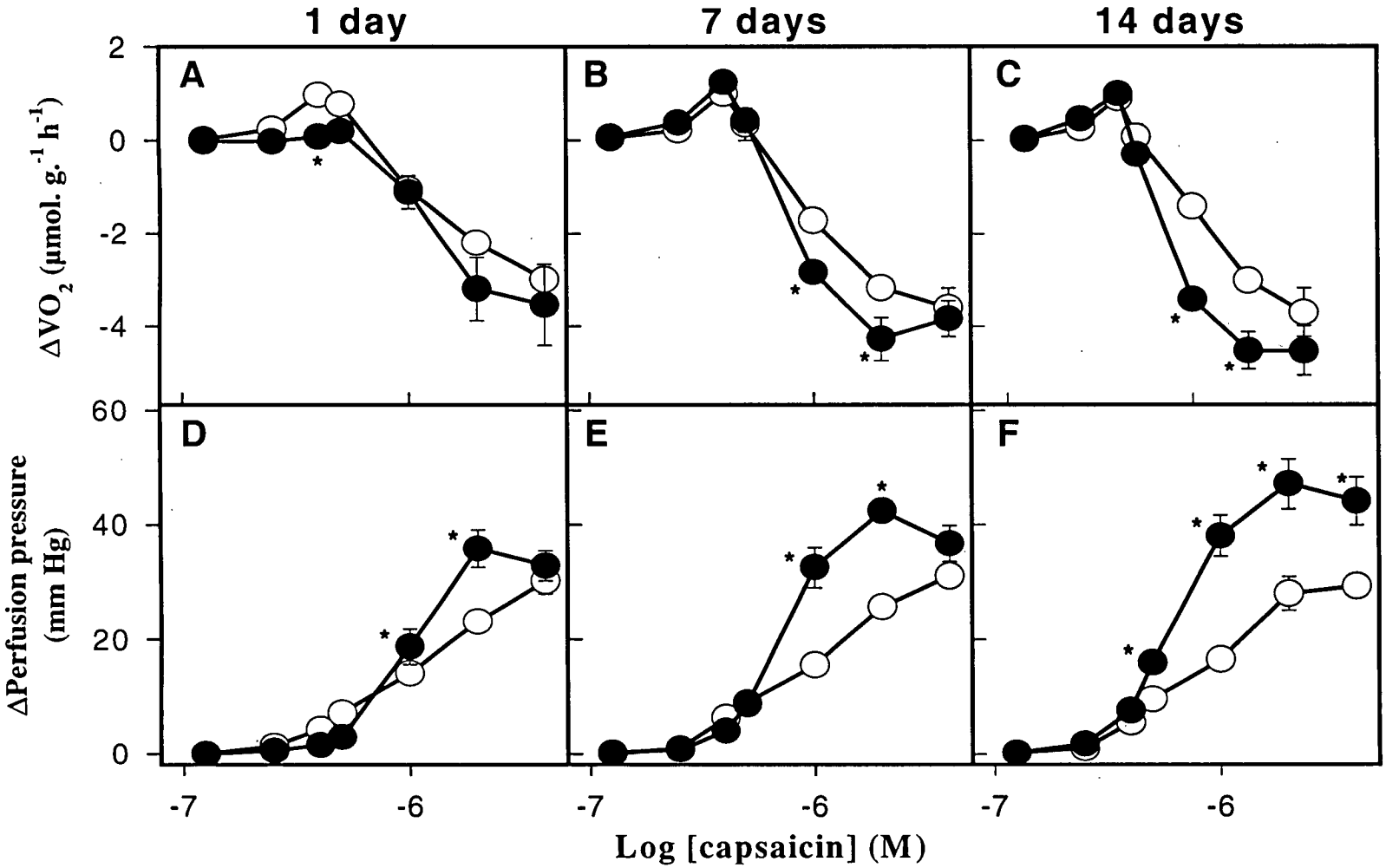


Fig. 3.5. Concentration-response curves for capsaicin-induced changes in oxygen consumption (A,B,C) and perfusion pressure (D,E,F) in the hindlimbs of rats perfused 1, 7 and 14 days after pretreatment with vehicle (○) or capsaicin (●). *P<0.05 from control (one-way ANOVA). Values are mean±s.e. mean in 4-6 experiments.

3.4. Discussion

To date, there have been few attempts to characterise the post-receptor mechanisms involved in the powerful vasoconstrictor and biphasic VO_2 responses induced by capsaicin in the perfused rat hindlimb (Cameron-Smith *et al.*, 1990; Colquhoun *et al.*, 1995; Chapter 2). Earlier studies have indicated the importance of external calcium and mitochondrial function in some of these responses (Colquhoun *et al.*, 1995), however the role of capsaicin sensitive primary afferent (sensory) neurons and sensory neuropeptides (SP, NKA, NKB and CGRP), and their receptors, has not been closely investigated until now. The data presented in this chapter has added to the understanding of the mechanisms involved in the acute effects of capsaicin in muscle, and has yielded some interesting and unexpected findings.

3.4.1. Role of the tachykinins

3.4.1.1. Non-peptide antagonists

The first report on the use of the selective NK1 receptor antagonist CP-99,994 showed this agent to possess picomolar affinity (K_i = 250 pM) for human NK1 receptors (IM-9 cells) labelled with [^{125}I]-BHSP (McLean *et al.*, 1993), and similar affinity (IC_{50} = 520 pM) for [^{3}H]-SP labelled NK1 receptors in the cat cortex (Lucot *et al.*, 1997). However, this compound exhibits only high micromolar affinity (IC_{50} = >10 μM) for NK2 ([^{125}I]-NKA-labelled hamster bladder) and NK3 ([^{125}I]-eledoisin-labelled guinea-pig cortex) receptors (McLean *et al.*, 1993). These authors also showed CP-99,994 to have greatly reduced potency for the non-specific inhibition of Ca^{2+} channels in rat heart (IC_{50} = 3-4 μM), compared to the parent non-peptide NK1 antagonist CP-96,345 (IC_{50} = 27 nM). Consequently, CP-99,994 represents a highly selective and powerful tool for the study of NK1 receptors in individual tissues.

Data presented in this chapter showed that the stimulation of VO_2 induced by submicromolar concentrations of capsaicin (VN_1 response) was partly blocked by the selective NK1 receptor antagonist CP-99,994 in a concentration-dependent manner (Fig 3.1) indicating a potential role for NK1 receptors in this response. Based on this data alone, an estimation of the potency (e.g. pA_2 or IC_{50}) of CP-99,994 for inhibiting the VO_2 response could not be made as insufficient concentrations of the antagonist were

used. Nonetheless, it appears from figure 3.1 that CP-99,994 possesses submicromolar to micromolar potency for inhibiting the effects of capsaicin in the hindlimb preparation, and differs from the *in vitro* affinity estimates of CP-99,994 for human and feline NK1 receptors reported above. However, the affinity estimates for CP-99,994 at rat NK1 receptors (IC_{50} = 127 nM) also differs markedly from that in the human and cat by at least 2-3 orders of magnitude (McLean and Lowe, 1994). Similarly, the affinity of this antagonist for NK1 receptors in the mouse (pA_2 = 6.06) is markedly reduced in comparison to the rat NK1 receptor antagonist RP 67580 (pA_2 = 8.41) (Allogho *et al.*, 1997). Furthermore, concentrations of CP-99,994 similar to those used in the present study have been used to selectively block NK1 receptors in a variety of functional assays including: the relaxant and contractile responses to various tachykinin agonists in the guinea-pig trachea (Figini *et al.*, 1996); electrically-evoked SP release in the rat spinal cord (Malcangio and Bowery, 1994); capsaicin-induced hyperalgesia in the rat knee joint (Davis and Perkins, 1996); and septide- and [Sar^9, $Met(O\text{-}2)^{11}$]-SP-induced dilatation of submucosal arterioles (Moore *et al.*, 1997).

In the rat hindlimb preparation, the NK2 receptor antagonist SR48968 produced similar effects to CP-99,994, but also enhanced the inhibition of VO_2 induced by micromolar concentrations of capsaicin (VN_2 response), and potentiated vasoconstriction over the entire capsaicin concentration range (Fig 3.2). This potentiation of capsaicin-induced vasoconstriction indicates that endogenously released tachykinins, acting via NK2 receptors, may be dilators of the perfused hindlimb vasculature. As for CP-99,994, the apparent potency of SR 48968 for producing these effects on capsaicin-mediated responses appears to be reduced compared to that in *in vitro* studies (Emonds-Alt *et al.*, 1992). However as for CP-99,994, similar concentrations of SR 48968 to those used in the present study have been used to inhibit the actions of capsaicin and NK2 receptor agonists in other tissue preparations (Lou *et al.*, 1993; Huber *et al.*, 1993; Maggi and Giuliani, 1996), while the inactive enantiomer for SR 48968 (SR 48965) is without effect (Maggi and Giuliani, 1996). Nonetheless, results obtained from the use of micromolar concentrations of these non-peptide antagonists should be interpreted with caution since SR 48968 has been shown to be a highly potent NK2 receptor antagonist with picomolar to low nanomolar affinity in several tissue preparations (Qian *et al.*, 1994; Advenier, 1995; Croci *et al.*, 1995), including NK2 receptor bioassays (Maggi *et al.*, 1993c). In addition, micromolar

concentrations of SR 48968 exhibit local anaesthetic (Wang *et al.*, 1994) and opioid receptor agonist properties (Martin *et al.*, 1993). SR 48968 also shows sub-micromolar affinity for cloned human, but not rat, NK3 receptors (Chung *et al.*, 1994), and both SR 48968 and CP-99,994 have been shown to interact with the phenylalkamine binding site on L-type voltage-sensitive Ca^{2+} channels (Lombet and Spedding, 1994). Nonetheless, the results presented in this chapter using the non-peptide tachykinin antagonists, whilst not conclusive, do suggest the possibility that the tachykinins and their receptors are involved in the responses mediated by capsaicin in perfused muscle. Further evidence for the involvement of these neurotransmitters was obtained by the use of tachykinin peptide agonists in this preparation.

3.4.1.2. Peptide agonists

Infusion of SP, NKA or NKB, in the presence of the neutral endopeptidase inhibitor phosphoramidon, induced mild, concentration-dependent vasoconstriction and stimulated VO_2 (Fig 3.4). The use of phosphoramidon in these studies was considered essential as neutral endopeptidase is a prime mediator in the degradation of tachykinins in skeletal muscle tissue (Russell *et al.*, 1996). In the perfused hindlimb, NKA was at least 10-fold more potent than SP at stimulating VO_2 and vasoconstriction (Table 3.2) and its activity is comparable to that in the NK2 receptor bioassay (rabbit pulmonary artery) described by Regoli *et al.* (1987). The potency of SP in the present study is at least 1000-fold lower than in the NK1 receptor bioassay (dog carotid artery) and more closely resembles its activity on NK2 receptors in the rabbit pulmonary artery (Regoli *et al.*, 1987). However, the use of BSA as an essential colloid in the perfused hindlimb preparation may account for the apparent low potency of SP as this protein is notorious for binding various agents. However, assuming that peptide binding, if any, by BSA occurs equally for all the tachykinins, the rank order of potency for these peptides in the rat hindlimb preparation was NKA>NKB>SP and supports the notion of NK2 receptor involvement. Nonetheless, a role for NK1 receptors cannot be excluded for several reasons including: a) CP-99,994 was effective at partly blocking capsaicin-induced stimulation of VO_2 (Fig. 3.1); b) NKA has a strong affinity for NK1 receptors (Regoli *et al.* ,1994); and c) autoradiographic studies indicate that NK1 receptors are present in hindlimb skeletal muscle (soleus), possibly on blood vessels (see Appendix 1). Although the NK3-preferring peptide, NKB, stimulated VO_2 and vasoconstriction in the

present study, it is unlikely that NK3 receptors play a role in the capsaicin-mediated effects in muscle since their peripheral distribution is limited (Mastrangelo *et al.*, 1987; Guard *et al.*, 1990).

Taken together, these findings using neuropeptide agonists and antagonists provide strong evidence that stimulation of VO_2 by submicromolar concentrations of capsaicin is partly mediated by the endogenous release of SP and NKA which then stimulate VO_2 via an action on peripheral NK2 receptors, and possibly NK1 receptors.

3.4.2. Role of CGRP

CGRP, which is released in skeletal muscle in response to capsaicin (Santicioli *et al.*, 1992), may act as a potent vasodilator in this preparation since the CGRP receptor antagonist $CGRP_{(8\text{-}37)}$ greatly potentiated the capsaicin-induced vasoconstriction and inhibition of VO_2 (Fig 3.3). However, infused CGRP (with phosphoramidon) did not produce a measurable effect on basal hindlimb VO_2 and vascular tone (Fig 3.4). This observation is unusual since CGRP has been shown to be a potent vasodilator in many tissues including striated muscle (White *et al.*, 1993; Kim *et al.*, 1995). In addition, it has recently been shown that CGRP, released from capsaicin-sensitive primary afferents, contributes partly to the hyperaemic response to skeletal muscle contraction (via sciatic nerve stimulation) in the rat hindlimb (Yamada *et al.*, 1997a; 1997b). However, basal hindlimb perfusion pressure in the present study probably represents near-maximum arteriolar dilatation since, at the flow rate used (4 ml. min^{-1}), the potent vasodilator nitroprusside has no measurable effect on vascular tone (Colquhoun *et al.*, 1988; Ye *et al.*, 1990). This may limit the scope of action of SP, NKA, NKB and/or CGRP and, therefore, any vasodilator action by these peptides would not be observed. Indeed, this possibility is examined in the studies presented in Chapter 4.

3.4.3. The role of capsaicin-sensitive neurons

Capsaicin possesses a well-documented ability to both stimulate and then desensitise peptide-containing sensory neurons (Holzer *et al.*, 1991; Szolcsányi, 1993; section *1.3.5.*). Indeed capsaicin is a widely used research tool that selectively blocks C-type and Aδ-type primary afferents. In the present studies, an attempt was made to define a role for capsaicin-sensitive neurons in the acute metabolic and vascular effects

of vanilloids in perfused muscle by studying the effects of systemic capsaicin pretreatment.

Capsaicin pretreatment produced dramatic changes in capsaicin-induced VO_2 and perfusion pressure changes in the perfused hindlimb (Fig 3.5). One day after capsaicin pretreatment, the stimulation of VO_2 and mild increase in perfusion pressure produced by submicromolar concentrations of capsaicin (VN_1 response) was almost completely abolished. However, 7 days after capsaicin pretreatment, the VN_1 response had returned and the magnitude of VO_2 stimulation was identical to that of the control. The acute sensory neuron block described by Szolcsányi (1993) (see section *1.3.5.1.*) may explain the absence of the VN_1 response 1 day after capsaicin-pretreatment. The re-establishment of the VN_1 response after 7 days may be due to a small population of intact C-fibres which recover from the block and release sufficient neuropeptides to stimulate VO_2.

In contrast to the effects of capsaicin pretreatment on VN_1 responses, the inhibition of VO_2 (VN_2 response) was marginally enhanced 1 day, and significantly enhanced 7 and 14 days after capsaicin pretreatment. A progressive increase in the vasoconstrictor response to capsaicin mirrored the enhancement of VO_2 inhibition, with maximum perfusion pressure to 2 μM capsaicin infusion almost doubling 14 days after capsaicin pretreatment. Further analysis of the data revealed that the concentration of capsaicin producing a half-maximal increase in perfusion pressure (EC_{50}) was significantly ($P<0.01$) decreased 7 and 14 days after capsaicin pretreatment (Table 3.3). Why the maximum vasoconstrictor response progressively increased in capsaicin pretreated rats is unclear. This was an unexpected finding since capsaicin-pretreatment normally leads to a blunting of non-vascular, smooth muscle responses to capsaicin (Maggi and Meli, 1988). This observation, when combined with the decrease in EC_{50} for capsaicin, suggests either up-regulation of VN_2 receptors and/or sensitisation of vascular smooth muscle to the constrictor action of capsaicin. Alternatively, the apparent increased sensitivity of the vasculature to the vasoconstrictor action of capsaicin may be due to the absence of sufficient vasodilator peptides (eg. CGRP) to counteract the receptor-mediated vasoconstrictor action of the vanilloid. Indeed denervation of cat cerebral arteries by "cold storage" (Saito *et al.*, 1988), or capsaicin pretreatment and trigemminal ganglionectomy (Edvinsson *et al.*, 1990) attenuates capsaicin-induced vasodilatation but not vasoconstriction, presumably by depleting

vasodilator peptides from sensory neurons. Similar results have been obtained in the guinea-pig thoracic aorta and carotid artery after capsaicin pretreatment where subsequent application the vanilloid induces vasoconstriction, rather than dilatation (Duckles, 1986).

A vasodilator role for endogenously released sensory peptides is not supported by the present findings where the infusion of SP, NKA and NKB resulted in a mild vasoconstrictor effect, and CGRP did not alter vascular tension. However, it remains to be seen whether or not SP, NKA and CGRP can vasodilate in the constant flow perfused hindlimb under vascular tension using a strong vasoconstrictor (eg. noradrenalin, serotonin, angiotensin II). The data presented in Chapter 4 explores this possibility.

Chapter 4

Effect of the Sensory Neuropeptides on the Perfused Rat Hindlimb Vascular Bed Preconstricted with Noradrenalin.

4.1 Introduction

In the results shown in Chapter 3, CGRP was without effect in the perfused rat hindlimb under basal conditions, an observation that was possibly due to the reduced basal vascular tone in the constant-flow preparation. Indeed, CGRP is renowned for its potent vasodilator effect, particularly with respect to its role in cutaneous neurogenic inflammation where the peptide is a prime mediator of vasodilatation in response to sensory nerve stimulation (reviewed by Brain, 1996; Holzer, 1998). In addition, CGRP has been shown to play an important role in inflammatory responses in the knee joint of the rat (Cambridge and Brain, 1992) and rabbit (Ferrell *et al.*, 1997) where it induces strong local vasodilatation that is attenuated by the CGRP antagonist $CGRP_{(8\text{-}37)}$ (McMurdo *et al.*, 1997), and may also act synergistically with other mediators to increase plasma extravasation (Cambridge and Brain, 1992). The ability of CGRP to relax blood vessels from a variety of species in various tissues, both *in vitro* and *in situ*, is further testament to the vasodilatory action of this peptide (reviewed by Poyner, 1992; Bell and McDermott, 1996). Furthermore, the *in vivo* administration of low doses of CGRP (0.1-10 nmol . kg^{-1}, i.v.) in the rat causes a reduction in mean arterial blood pressure, increased heart rate and cardiac output, and decreased resistance in the vascular beds of the kidney, mesentery and hindquarter (Sirén and Feuerstein, 1988). Of particular interest to the present study is the latter observation by Sirén and Feurerstein (1988) which demonstrates the ability of CGRP to induce dilatation in a skeletal muscle vascular bed. Indeed others have shown a similar response to CGRP in skeletal muscle tissue (White *et al.*, 1993; Kim *et al.*, 1995; Didion and Mayhan, 1997), including that of the rat hindlimb (Yamada *et al.*, 1997a; 1997b).

Under conditions of reduced basal vascular tone, the tachykinins SP, NKA and NKB are mild vasoconstrictors in the perfused rat hindlimb and induce a concomitant increase in muscle oxygen consumption (Chapter 3, Fig. 3.4). SP, NKA and NKB

display similar effects on vascular tone in rabbit isolated pulmonary artery segments where, in the absence of endothelium, these peptides induce concentration-dependent vasoconstriction by the stimulation of tachykinin (possibly NK2) receptors on vascular smooth muscle (D'Orléans-Juste *et al.*, 1985). A more recent study by Shirahase *et al.* (1995) further supports the involvement of smooth muscle NK2 receptors where the endothelium-independent constriction of isolated rabbit pulmonary artery by submicromolar concentrations of SP was inhibited by SR 48968, but not by the NK1 antagonists CP-96,345, CP-99,994, and SR-140,333. Interestingly, these authors also showed endothelium-dependent constriction of these vessels by nanomolar concentrations of SP acting via NK1 receptors. Similarly, an NK1-mediated vasoconstrictor action in response to tachykinins has been reported in the rabbit jugular vein (Nantel *et al.*, 1990). Hence, vasoconstriction by these peptides is not strictly limited to the stimulation of smooth muscle tachykinin (NK2) receptors.

The studies presented in Chapter 3, where the hindlimb preparation was essentially fully dilated, further support the notion of tachykinin-induced vasoconstriction by the stimulation of smooth muscle NK2 receptors, since the rank order of potency for the tachykinins in these experiments was NKA>NKB>SP. However, the tachykinins, most notably SP, are better known for their potent vasodilator action via the stimulation of NK1 receptors on the endothelium (Regoli *et al.*, 1987; Couture *et al.*, 1989; Stewart-Lee and Burnstock, 1989; Delay-Goyet *et al.*, 1992). Similar observations have been made in skeletal muscle preparations in the rat (Brock and Joshua, 1991; Huang and Koller, 1996), rabbit (Persson *et al.*, 1991; Gustafsson *et al.*, 1994), hamster (Hall and Brain, 1994) and human (Casino *et al.*, 1995; Newby *et al.*, 1997b).

Given the strong vasodilator nature of CGRP and the tachykinins in skeletal muscle and other preparations (see above), and since the tachykinins are able to vasoconstrict via stimulation of endothelial NK1 or smooth muscle NK2 receptors, the aim of the present chapter was to examine the effect of these peptides in the perfused rat hindlimb under near-physiological vascular tone. Increased vascular tone was achieved by the use of the vasoconstrictors noradrenalin (NOR) or 5-hydroxytryptamine (serotonin, 5-HT), the actions of which have been well characterised in this preparation (reviewed by Clark *et al.*, 1995; 1997). These experiments were designed to add to the current knowledge of neuropeptide action in the unstimulated perfused rat hindlimb

where they either vasoconstrict (with an increase in VO_2) or do little to alter vascular tone (see Chapter 3, Fig. 3.4). In addition, an attempt was made to assess the involvement of nitric oxide (NO) production, by nitric oxide synthase (NOS), in the vasodilator responses to neuropeptides. This was achieved by the infusion of CGRP, SP and NKA in the presence of Nω-nitro-L-arginine methyl ester (L-NAME), a known NOS inhibitor (Rees *et al.*, 1990), in the perfused hindlimb preparation stimulated with NOR. The ability of this inhibitor to block NO production was assessed by co-infusing it with the NO-dependent vasodilator carbamyl choline (carbachol or CCh). Furthermore, the effect of low and high concentrations of capsaicin were examined in this preparation under NOR-induced vascular tone to determine the actions of this sensory nerve stimulant under near-physiological pressures.

4.2. Methods

4.2.1. Rat hindlimb perfusion.

Animals were anaesthetised with pentobarbitone sodium (60 mg/kg) and their left hindlimbs perfused according to the method described previously (section *2.2.1*, Ruderman *et al.*, 1971; Colquhoun *et al.*, 1988). Hindlimb vascular tone (perfusion pressure) was increased by the infusing 0.6 μM NOR or 0.8 μM 5-HT after basal steady state conditions were attained (approximately 30 minutes). The infusion of other agents did not commence until steady state vasoconstrictor-stimulated VO_2 and perfusion pressure were reached.

4.2.2. Materials

Substance P, neurokinin A, and calcitonin gene-related peptide were purchased from Auspep (Australia); capsaicin, carbamyl choline (carbachol or CCh), 5-hydroxytryptamine (serotonin or 5-HT), Nω-nitro-L-arginine methyl ester (L-NAME), noradrenalin (NOR), Sigmacote® and phosphoramidon from the Sigma Chemical Company; bovine serum albumin (Fraction V) from Boehringer Mannheim (Australia), pentobarbitone sodium (Nembutal, 60 mg/ml) from Bomac Laboratories (Australia), and heparin sodium (5000 $IU.ml^{-1}$) from David Bull Laboratories (Australia). All other reagents were of analytical grade.

Tachykinins were dissolved into 20 µl aliquots using a 0.01 M acetic acid solution containing 1% β-mercaptoethanol and stored at -20°C to maintain chemical stability. The aliquots were then diluted, as needed, with 0.9% NaCl so that the acetate and β-mercaptoethanol concentrations were negligible. Due to the lipophilic nature of capsaicin, it was dissolved in 50% ethanol; thus care was taken to keep the infusion rates low (usually below 10 µl/min) to avoid vehicle-induced effects. All other agents were dissolved in normal saline (0.9% w/v).

4.2.3 Agent infusion

Infusion of all agents occurred by the use of methods and equipment described in sections *2.2.4* and *3.2.3*. In perfusions using SP, NKA or CGRP, all glassware in contact with these peptides was thoroughly cleaned with a strong alkaline solution (1M NaOH), rinsed and silanised with Sigmacote® (prior to infusion) to prevent peptide adhesion to glass surfaces.

4.2.3.1. Neuropeptide experiments

For experiments with the neuropeptides, NOR (0.6 µM) or 5-HT (0.8 µM) were first infused during steady state basal venous PO_2 (and therefore VO_2) and perfusion pressure. After the establishment of a new steady state VO_2 and vascular tone, the neutral endopeptidase inhibitor phosphoramidon (5 µM) was co-infused with either vasoconstrictor. In experiments using NOR, phosphoramidon caused mild dilatation that was overcome by increasing the NOR concentration to 0.8 µM. Either SP (50 nM), NKA (20 nM) or CGRP (20 nM) were then infused until steady state conditions were again observed. In separate experiments, L-NAME (10 µM) was infused after phosphoramidon. The protocol was otherwise identical to the control experiments described above in the absence of L-NAME.

4.2.3.2. Carbachol experiments

For experiments using CCh, NOR (0.6 µM) was first infused and new steady state VO_2 and perfusion pressure were attained. A submicromolar concentration of CCh (0.5 µM) was co-infused with NOR for at least 20-30 minutes to fully observe the effects of this acetylcholine mimetic. All drugs were then removed, and the hindlimb preparation was allowed to recover to basal VO_2 and perfusion pressure. In the same experiment,

the above protocol was repeated with the addition of L-NAME (10 μM) prior to the infusion of CCh to observe any effects that the NOS inhibitor alone may have on NOR-induced VO_2 and perfusion pressure changes. CCh was infused for at least 45 minutes to observe its overall effect in the presence of L-NAME.

4.2.3.3. Capsaicin experiments

In the experiments with capsaicin a similar protocol to those with CCh was used in that NOR (0.6 μM) was first infused followed by capsaicin after the achievement of NOR-stimulated steady state VO_2 and perfusion pressure. A low concentration of capsaicin (50 nM) was co-infused with NOR, followed by the testing of a higher capsaicin concentration (0.5 μM). Both capsaicin concentrations were infused until the re-establishment of steady state VO_2 and perfusion pressure.

4.3.4. Data analysis

Statistical analysis was performed using Student's t-test, one-way analysis of variance (ANOVA) or ANOVA on ranks (Kruskal-Wallis analysis), or by one-way repeated measures ANOVA or one-way repeated measures ANOVA on ranks (Friedman analysis), where applicable. All ANOVAs were subjected to Student-Newman-Keuls multiple comparisons. $P<0.05$ was considered statistically significant.

4.3 Results

For all data presented in this chapter, the basal VO_2 and perfusion pressure values, as well as those for all concentrations of NOR and 5-HT, are presented in Table 4.1. The concentrations of NOR and 5-HT used (0.6-0.8 μM) were chosen to give approximately physiological hindlimb perfusion pressure values under steady state conditions (90-120 mm Hg). These vasoconstrictors also induced a concomitant stimulation (NOR) or reduction (5-HT) in hindlimb VO_2 similar to that seen previously in this preparation (reviewed by Clark *et al.*, 1995; 1997).

TABLE 4.1. Basal and serotonin (5-HT)- or noradrenalin (NOR)-stimulated oxygen consumption (VO_2) and perfusion pressure (PP) in the perfused rat hindlimb.

Parameter	n	VO_2 (μmol. $g.^{-1}$ h^{-1})	PP (mm Hg)
Basal	37	9.22 ± 0.10	28.49 ± 0.50
0.6 μM NOR	27	12.76 ± 0.10	94.30 ± 2.65
0.8 μM NOR + 5 μM phosphoramidon	25	12.86 ± 0.10	95.00 ± 2.39
0.8 μM 5-HT	4	5.55 ± 0.19	106.50 ± 6.40

All values are mean ± s.e. mean steady state VO_2 or PP.

4.3.1. Effect of calcitonin gene-related peptide (CGRP) in serotonin (5-HT)-stimulated perfused hindlimb.

The infusion of 5-HT (0.8 μM) into the perfused rat hindlimb induced a characteristic inhibition of VO_2 (ΔVO_2, -2.85±0.27 μmol. $g.^{-1}$ h^{-1}) coupled with strong vasoconstriction (ΔPP, 75.8±7.0 mm Hg) (Fig. 4.1). The subsequent infusion of the neutral endopeptidase inhibitor phosphoramidon (5 μM) did not have a statistically significant ($P>0.05$, Table 4.2) effect on either 5-HT-induced VO_2 or vascular effects (ΔVO_2, -2.73±0.27 μmol. $g.^{-1}$ h^{-1}; ΔPP, 74.3 ± 8.4 mm Hg).

The co-infusion of CGRP (20 nM) with 5-HT and phosphoramidon caused a small reversal of 5-HT-induced inhibition of VO_2 (ΔVO_2, -2.35±0.15 μmol. $g.^{-1}$ h^{-1}) and mild dilatation (ΔPP, 59.8 ± 9.6 mm Hg). The VO_2 and vascular changes induced by CGRP were reproducible and showed statistical significance by repeated measures ANOVA and Student-Newman-Keuls comparisons ($P<0.05$, Table 4.2).

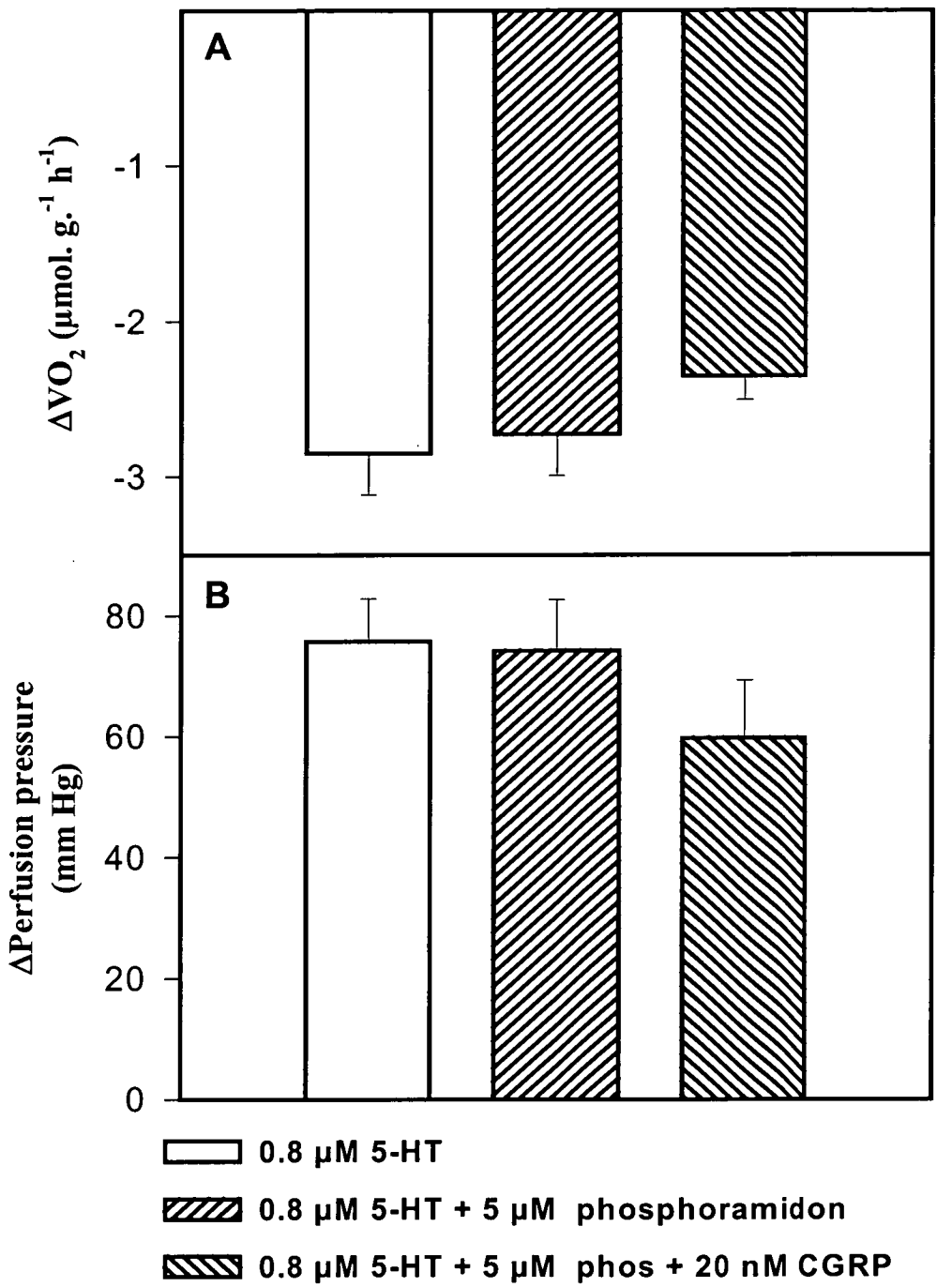


Fig. 4.1. Effect of calcitonin gene-related peptide (CGRP) on serotonin (5-HT)-induced oxygen consumption (VO_2) (panel A) and perfusion pressure (panel B) changes in the perfused rat hindlimb (n = 4). Statistical analysis of the data by one-way repeated measures ANOVA and pairwise comparisons (Student-Newman-Keuls method) is presented in Table 4.2.

TABLE 4.2. One-way repeated measures analysis of variance (ANOVA) and pairwise multiple comparisons (Student-Newman-Keuls method) of the data presented in Fig. 4.1 for CGRP-induced changes in oxygen consumption (ΔVO_2), and perfusion pressure (ΔPP).

Test	ΔVO_2	ΔPP
ANOVA P value	0.047	0.003
F statistic	5.33	18.14
[1]S-N-K comparisons:		
5-HT vs. phosphoramidon	ns	ns
5-HT vs. CGRP	*	*
5-HT + phos vs. CGRP	ns	*

[1]Student-Newman-Keuls P value significant at P<0.05, * = significant, ns = not significant.

4.3.2 Effects of calcitonin gene-related peptide (CGRP) in noradrenalin (NOR)-stimulated perfused hindlimb

4.3.2.1. Time course for CGRP-induced effects

The infusion of NOR (0.6 μM) into the perfused rat hindlimb preparation results in a rapid decrease in venous PO_2 (increase in hindlimb VO_2) and a concomitant increase in arterial perfusion pressure (indicative of vasoconstriction) (Fig. 4.2A, B). Both venous PO_2 and perfusion pressure reached steady state before the infusion of phosphoramidon (5 μM) which did little to alter the NOR-induced venous PO_2 change, but induced mild dilatation (decrease in perfusion pressure). The infusion of CGRP (20 nM), in the presence of NOR and phosphoramidon, caused strong dilatation that was sustained until the removal of the peptide (Fig. 4.2B). In addition, the reduction in venous PO_2 produced by NOR was partly reversed by CGRP, and this effect was also sustained for the duration of the peptide infusion (Fig. 4.2A). The infusion of CGRP concentrations greater than 20 nM did not cause stronger dilatation or reversal of NOR-induced venous PO_2 (VO_2) effects, while picomolar concentrations did little to NOR-induced effects (data not shown). Hence 20 nM CGRP was considered to be a near-maximal effective concentration.

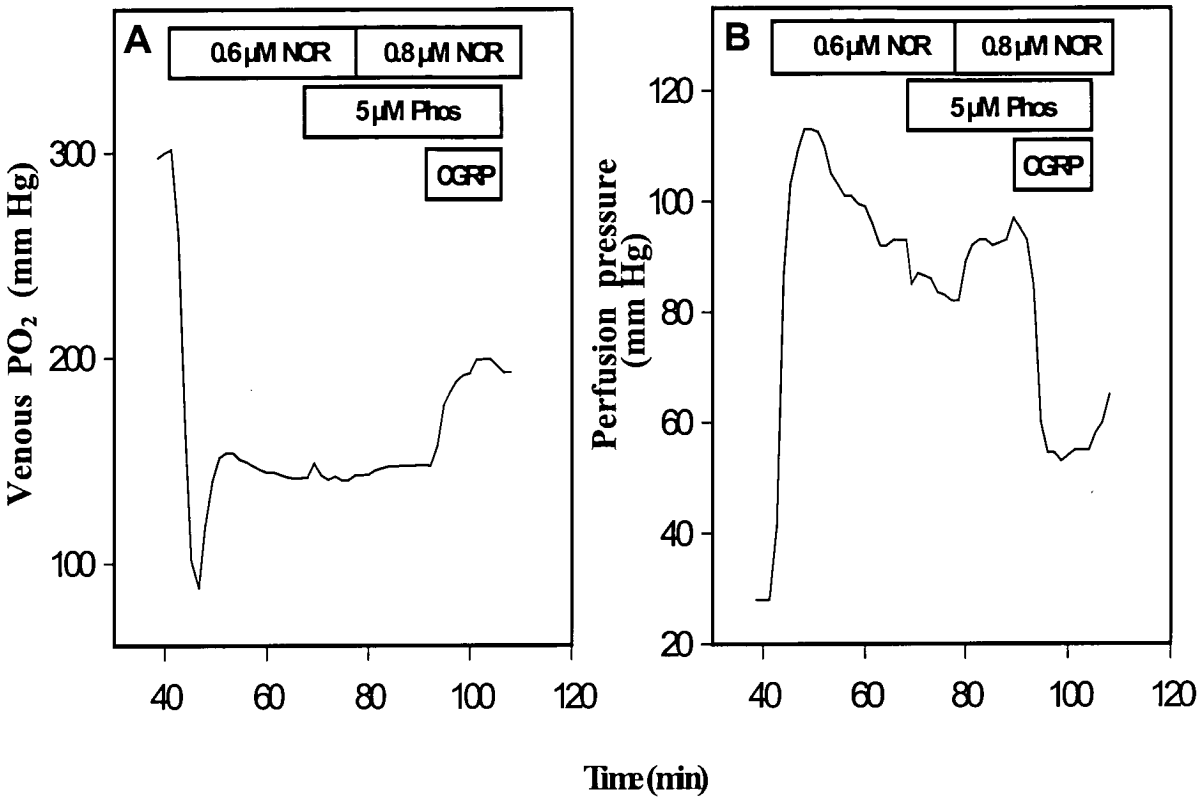


Fig. 4.2. Typical tracing of the actions of 20 nM calcitonin gene-related peptide (CGRP) and phosphoramidon (phos) on venous partial pressure of oxygen (PO_2) (panel A) and arterial perfusion pressure (panel B), in the perfused rat hindlimb stimulated with noradrenalin (NOR).

4.3.2.2. CGRP-induced effects and the importance of nitric oxide formation

A submicromolar concentration of NOR (0.6 μM) stimulated basal hindlimb VO_2 (ΔVO_2, 3.45 ± 0.13 μmol. $g.^{-1}$ h^{-1}, Fig. 4.3A) and produced a marked increase in perfusion pressure (ΔPP, 59.5 ± 3.8 mm Hg, Fig. 4.3C). In contrast to the data obtained using 5-HT as the vasoconstrictor, the infusion of phosphoramidon (also 5 μM) caused a small but statistically significant dilatation ($P<0.05$, Table 4.3), but had no apparent effect on NOR-induced stimulation of VO_2 (ΔVO_2, 3.33 ± 0.21 μmol. $g.^{-1}$ h^{-1}; ΔPP, 44.3 ± 2.7 mm Hg Figs. 4.2 and 4.3A, C). Due to the unexpected vascular effects of phosphoramidon, the concentration of NOR was increased to 0.8 μM to restore

perfusion pressure (ΔPP, 57.8 ± 2.8 mm Hg), with no change in VO_2 (ΔVO_2, 3.33 ± 0.21 μmol. $g.^{-1}$ h^{-1}, Fig. 4.2 and 4.3).

The subsequent infusion of CGRP (20 nM) caused a significant ($P<0.05$, Table 4.3) reversal of the NOR-induced perfusion pressure increase, indicative of vasodilatation (ΔPP, 27.0 ± 1.1 mm Hg, Fig. 4.3C), and reversed NOR-induced stimulation of VO_2 (ΔVO_2, 2.23 ± 0.28 μmol. $g.^{-1}$ h^{-1}, Fig. 4.3A).

In a separate set of perfusions, the above protocol was repeated but with the addition of the nitric oxide synthase inhibitor L-NAME (10 μM). Again, the infusion of NOR (0.6 μM) induced strong stimulation of VO_2 (ΔVO_2, 3.40 ± 0.20 μmol. $g.^{-1}$ h^{-1}, Fig. 4.3B) and increased perfusion pressure (ΔPP, 63.0 ± 1.0 mm Hg). However, in this set of experiments the co-infusion of phosphoramidon (also 5 μM) did not significantly ($P>0.05$, Table 4.3) alter vascular tone (ΔPP, 61.5 ± 2.5 mm Hg) (Fig. 4.3D). The infusion of L-NAME, for approximately 10 minutes prior to the infusion of CGRP, had no apparent effect on NOR-induced VO_2 or perfusion pressure changes ($P>0.05$, Table 4.3; data not shown in Fig 4.3). Due to the apparent lack of dilatation by phosphoramidon, increasing the concentration of NOR to 0.8 μM raised the perfusion pressure above that seen with the infusion of 0.6 μM NOR (ΔPP, 82.0 ± 6.4 mm Hg, Fig. 4.3D), but did little to further alter VO_2 (ΔVO_2, 3.33 ± 0.16 μmol. $g.^{-1}$ h^{-1}, Fig. 4.3B). The infusion of CGRP (20 nM) into the perfused hindlimb preparation caused a significant ($P<0.05$, Table 4.3) reversal of NOR-induced VO_2 effects (ΔVO_2, 2.13 ± 0.38 μmol. $g.^{-1}$ h^{-1}, Fig. 4.3B) and strong vasodilatation (ΔPP, 32.5 ± 1.8 mm Hg, Fig. 4.3D), very similar to that seen in the absence of L-NAME (Fig. 4.3A, C).

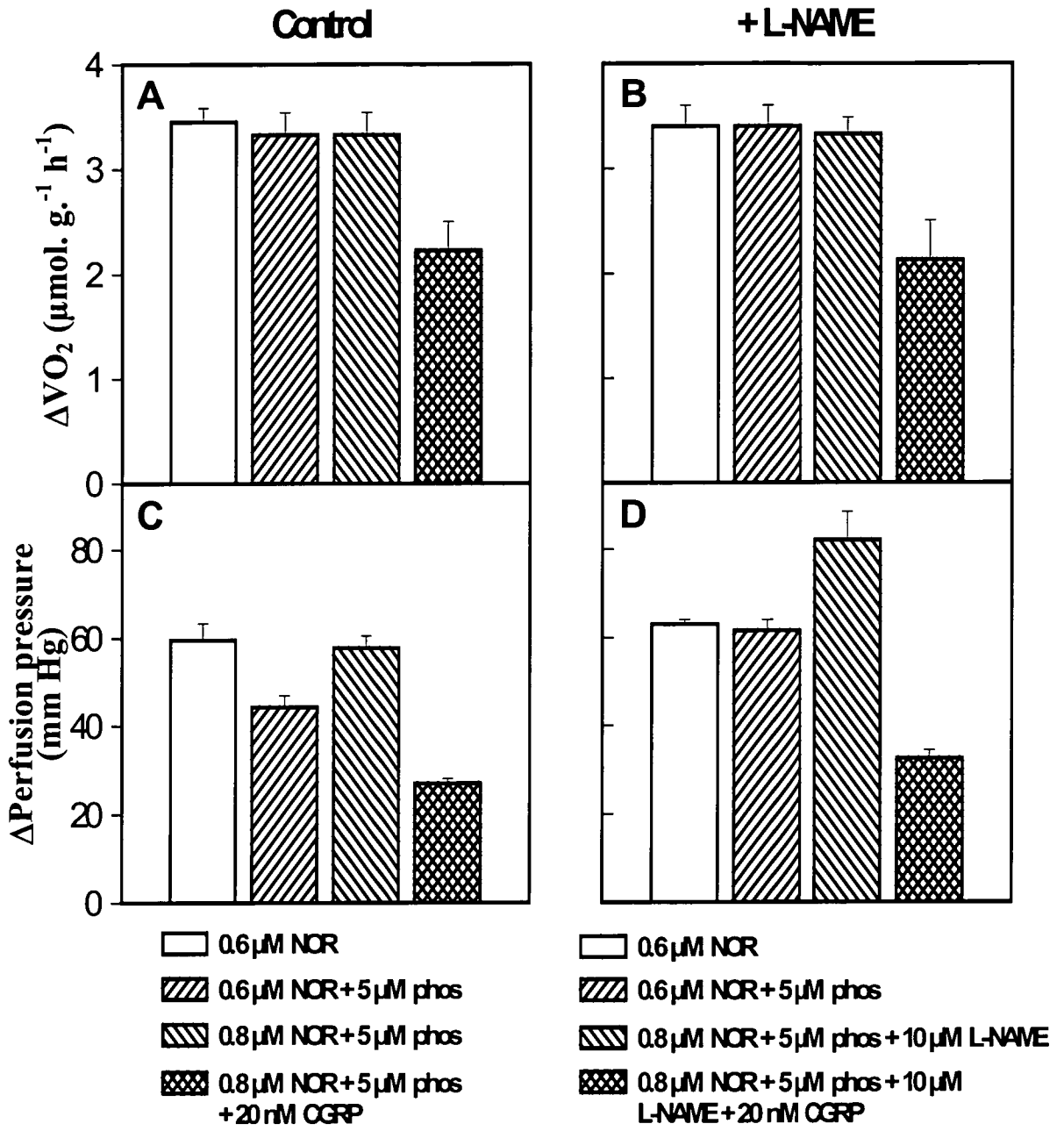


Fig. 4.3. Effect of calcitonin gene-related peptide (CGRP) and phosphoramidon (phos) on noradrenalin (NOR)-induced oxygen consumption (top panels) and perfusion pressure (bottom panels) changes in the perfused rat hindlimb (n = 4). Responses to CGRP were examined in the absence (panels A and C) and presence (panels B and D) of the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). Statistical analysis of the data by one-way repeated measures ANOVA and pairwise comparisons (Student-Newman-Keuls method) is presented in Table 4.3.

TABLE 4.3. One-way repeated measures analysis of variance (ANOVA) and pairwise multiple comparisons (Student-Newman-Keuls method) of the data presented in Fig. 4.3 for CGRP-induced changes in oxygen consumption (ΔVO_2), and perfusion pressure (ΔPP) in the absence and presence of L-NAME.

Test	Figure	ΔVO_2	ΔPP
[1]ANOVA P value	4.3A, C	<0.001	<0.001
F statistic	4.3A, C	65.64	58.54
[1]ANOVA P value	4.3B, D	0.002	<0.001
F statistic	4.3B, D	8.24	27.79
[2]S-N-K comparisons:			
[3]NOR vs. NOR + phos	4.3A, C	ns	*
[4]NOR + phos vs. CGRP + NOR + phos	4.3A, C	*	*
[3]NOR vs. NOR + phos	4.3B, D	ns	ns
[3]NOR + phos vs. L-NAME + NOR + phos	-	ns	ns
[4]NOR + phos + L-NAME vs. CGRP + NOR + phos + L-NAME	4.3B, D	*	*

[1]ANOVA P value significant at P<0.05

[2]Student-Newman-Keuls P value significant at P<0.05, * = significant, ns = not significant.

[3]NOR concentration = 0.6 μM

[4]NOR concentration = 0.8 μM

4.3.3 Effect of substance P (SP) in noradrenalin-stimulated perfused hindlimb

4.3.3.1. Time course for SP-induced effects

Due to the mild CGRP-induced dilatation in the 5-HT-stimulated perfused hindlimb, and the strong dilatation by this peptide in NOR-stimulated hindlimb, it was decided that the latter vasoconstrictor was more appropriate for providing the necessary vascular tone to examine the actions of other neuropeptides in this preparation. Hence, Figure 4.4 shows a typical tracing for the effects of SP on VO_2 and perfusion pressure changes induced by NOR in a single hindlimb perfusion experiment. As for some experiments with CGRP, the infusion of phosphoramidon caused mild vasodilatation

that was overcome by increasing the concentration of NOR to 0.8 μM. The infusion of 50 nM SP induced a transient reversal of VO_2 stimulation by NOR, and a rapid but transient vasodilator effect. While still under SP stimulation, both VO_2 and perfusion pressure returned to values approximately equal to that seen with the infusion of 0.8 μM NOR and phosphoramidon alone. In a preliminary experiment using concentrations of SP that were lower or higher than that used in the present study, it was concluded that

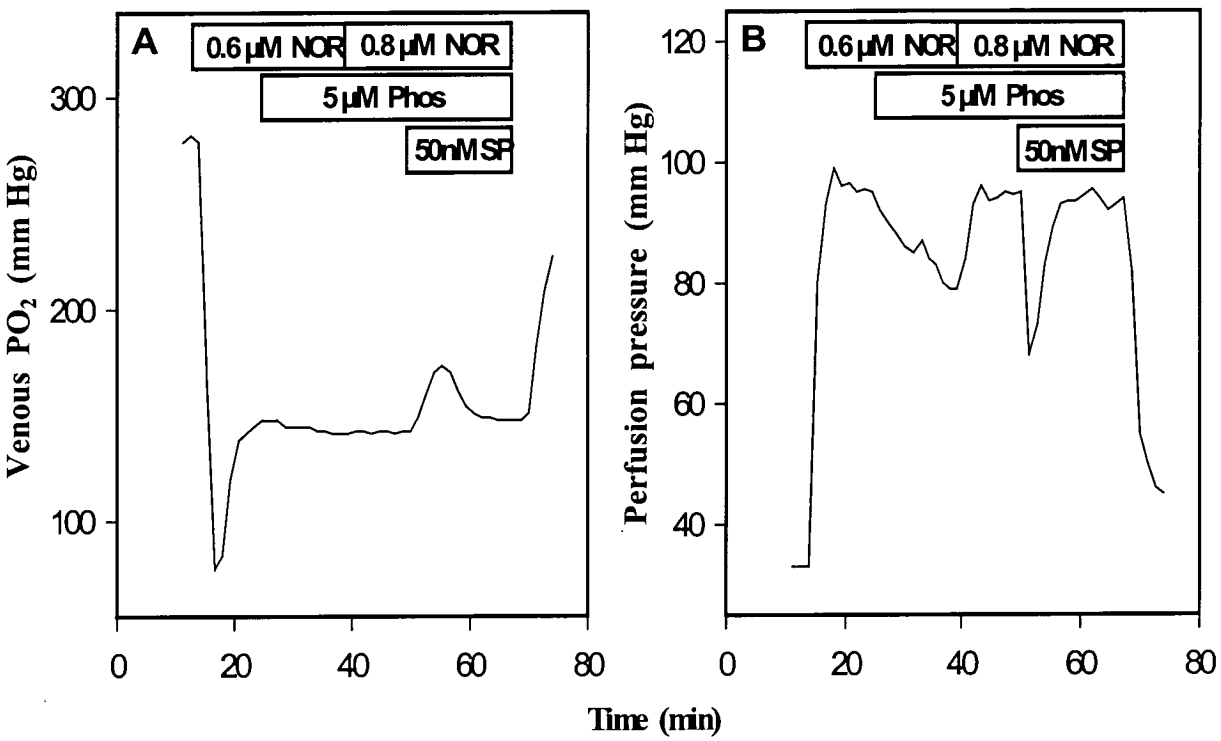


Fig. 4.4. Typical tracing of the peak and steady state actions of 50 nM substance P (SP) on venous partial pressure of oxygen (PO_2) (panel A) and arterial perfusion pressure (panel B), under noradrenalin (NOR) stimulation, in the perfused rat hindlimb.

50 nM SP induced near-maximal VO_2 and vascular responses (data not shown). Hence this concentration of the peptide was used in the remaining studies in the absence or presence of L-NAME.

4.3.3.2. SP-induced effects and the importance of nitric oxide formation

In several repeat experiments of the data shown in Figure 4.4, NOR (0.6 μM) stimulated basal hindlimb VO_2 (ΔVO_2, 3.44 ± 0.19 μmol. $g.^{-1}$ h^{-1}, Fig. 4.5A) and raised perfusion pressure (ΔPP, 61.4 ± 3.3 mm Hg, Fig. 4.5C) in a manner very similar to the data presented in section *4.3.2.1.* Again, phosphoramidon (5 μM) did not alter NOR-induced VO_2 changes, but caused mild dilatation (ΔPP, 50.8 ± 3.4 mm Hg) that was overcome by increasing the concentration of NOR to 0.8 μM (ΔVO_2, 3.38 ± 0.22 μmol. $g.^{-1}$ h^{-1}; ΔPP, 62.4 ± 4.1 mm Hg). The subsequent infusion of SP (50 nM) significantly ($P<0.05$, Table 4.4) reversed NOR-induced stimulation of VO_2 (peak ΔVO_2, 2.56 ± 0.25 μmol. $g.^{-1}$ h^{-1}) and dilated the hindlimb vasculature (peak ΔPP, 36.0 ± 2.1 mm Hg). However, these effects were not sustained throughout the duration of SP infusion and gradually returned to the steady state VO_2 and perfusion pressure values seen before peptide infusion (ΔVO_2, 3.28 ± 0.16 μmol. $g.^{-1}$ h^{-1}; ΔPP, 60.0 ± 4.2 mm Hg).

Repetition of the experiments just described in the presence of L-NAME (10 μM) yielded similar, but not identical results (Fig. 4.5B, D). Again, the NOR-induced perfusion pressure (ΔPP, 54.0 ± 2.7 mm Hg, Fig. 4.5D), but not VO_2 (ΔVO_2, 3.55 ± 0.16 μmol. $g.^{-1}$ h^{-1}, Fig. 4.5B), changes were significantly ($P<0.05$, Table 4.4) altered by phosphoramidon (ΔVO_2, 3.53 ± 0.13 μmol. $g.^{-1}$ h^{-1}; ΔPP, 46.5 ± 3.1 mm Hg). The co-infusion of L-NAME with phosphoramidon and NOR did not further alter VO_2 or perfusion pressure (ΔVO_2, 3.45 ± 0.16 μmol. $g.^{-1}$ h^{-1}; ΔPP, 46.0 ± 3.3 mm Hg). A higher concentration of NOR (0.8 μM) was again used to restore vascular tension, but had no greater effect on VO_2 (ΔVO_2, 3.48 ± 0.16 μmol. $g.^{-1}$ h^{-1}; ΔPP, 60.3 ± 3.6 mm Hg). The subsequent infusion of SP (50 nM) induced a statistically significant ($P<0.05$, Table 4.4) peak reversal of NOR-induced VO_2 and perfusion pressure changes very similar to that seen in the absence of L-NAME (peak ΔVO_2, 2.8 ± 0.04 μmol. $g.^{-1}$ h^{-1}; peak ΔPP, 35.8 ± 1.3 mm Hg). Similar to previous experiments, the effect of SP on VO_2 was not sustained (ΔVO_2, 3.28 ± 0.08 μmol. $g.^{-1}$ h^{-1}), nor was that on perfusion pressure which, interestingly, plateaued at a level above that seen with the infusion of NOR (0.8 μM), phosphoramidon and L-NAME alone (ΔPP, 69.5 ± 5.9 mm Hg). This increase in steady state perfusion pressure by SP in the presence of L-NAME, from that induced by 0.8 μM NOR, was statistically significant by repeated measures analysis of variance and subsequent Student-Newman-Keuls analysis ($P<0.05$, Table 4.4).

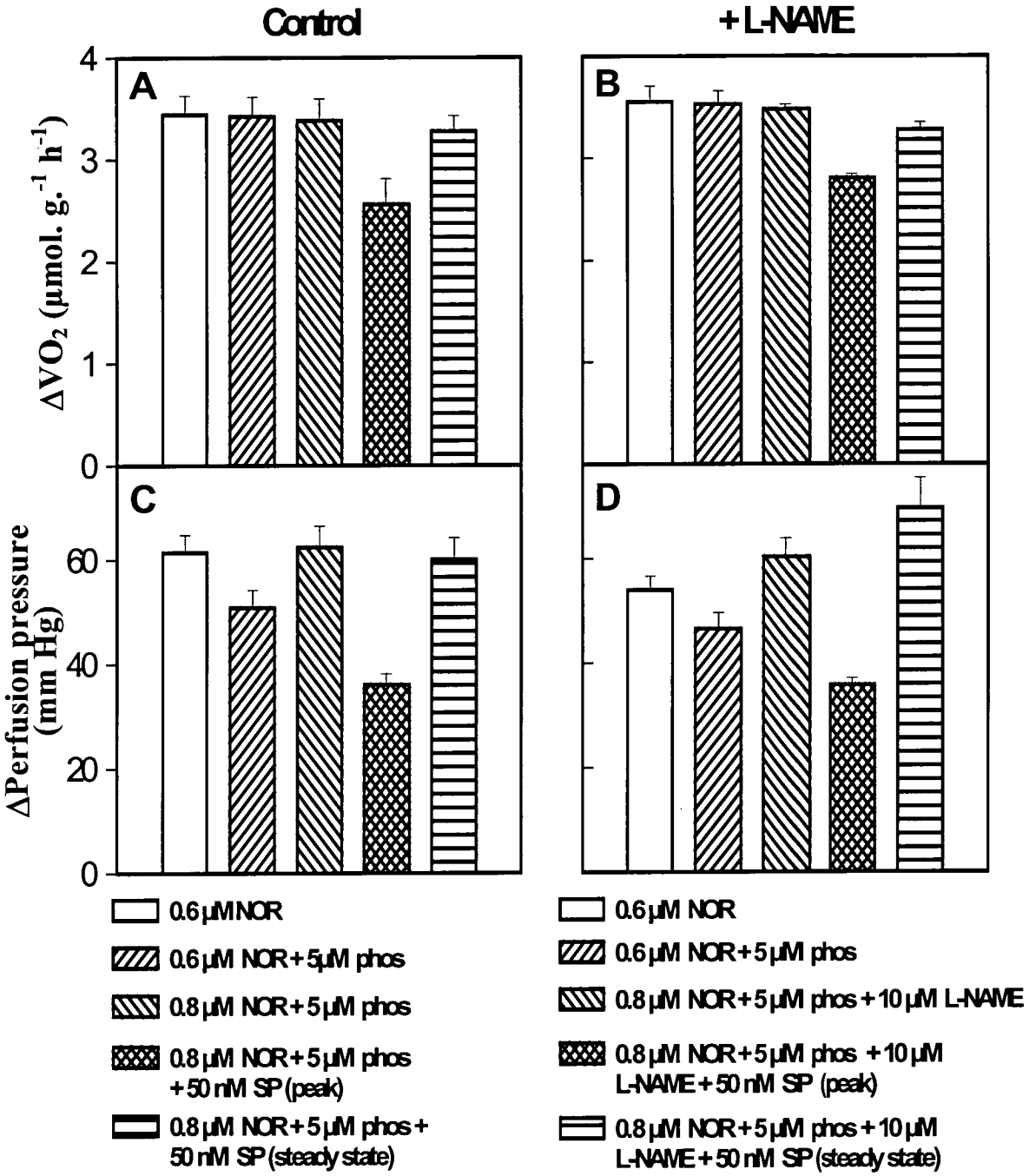


Fig. 4.5. Effect of substance P (SP) and phosphoramidon (phos) on noradrenalin (NOR)-induced oxygen consumption (top panels) and perfusion pressure (bottom panels) changes in the perfused rat hindlimb (n = 4-5). Responses to SP were examined in the absence (panels A and C) and presence (panels B and D) of the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). Statistical analysis of the data by one-way repeated measures ANOVA and pairwise comparisons (Student-Newman-Keuls method) is presented in Table 4.4.

TABLE 4.4. One-way repeated measures analysis of variance (ANOVA) and pairwise multiple comparisons (Student-Newman-Keuls method) of the data presented in Fig. 4.5 for SP-induced changes in oxygen consumption (ΔVO_2), and perfusion pressure (ΔPP) in the absence and presence of L-NAME.

Test	Figure	ΔVO_2	ΔPP
[1]ANOVA P value	4.5A, C	<0.001	<0.001
F statistic	4.5A, C	29.41	23.36
[1]ANOVA P value	4.5B, D	<0.001	<0.001
F statistic	4.5B, D	14.50	28.02
[2]S-N-K comparisons:			
[3]NOR vs. NOR + phos	4.5A, C	ns	*
[4]NOR + phos vs. SP (peak) + NOR + phos	4.5A, C	*	*
[4]NOR vs. SP (steady state)+ NOR + phos	4.5A, C	ns	ns
[3]NOR vs. NOR + phos	4.5B, D	ns	*
[3]NOR + phos vs. L-NAME + NOR + phos	-	ns	ns
[4]NOR + L-NAME + phos vs. SP (peak) + NOR + L-NAME + phos	4.5B, D	*	*
[4]NOR + L-NAME + phos vs. SP (steady state)+ NOR + L-NAME + phos	4.5B, D	ns	*

[1]ANOVA P value significant at P<0.05

[2]Student-Newman-Keuls P value significant at P<0.05, * = significant, ns = not significant.

[3]NOR concentration = 0.6 μM

[4]NOR concentration = 0.8 μM

4.3.4 Effect of neurokinin A (NKA) in noradrenalin-stimulated perfused hindlimb

4.3.4.1. Time course for NKA-induced effects

A typical tracing of the effects of NKA in a single perfused hindlimb experiment is presented in Figure 4.6. A low concentration of NKA (20 nM) caused alteration of the NOR-induced VO_2 and perfusion pressure responses, similar to the effects seen with SP. As for SP and CGRP, the concentration of NKA chosen was maximally effective since higher concentrations did not induce further changes. NKA transiently reversed

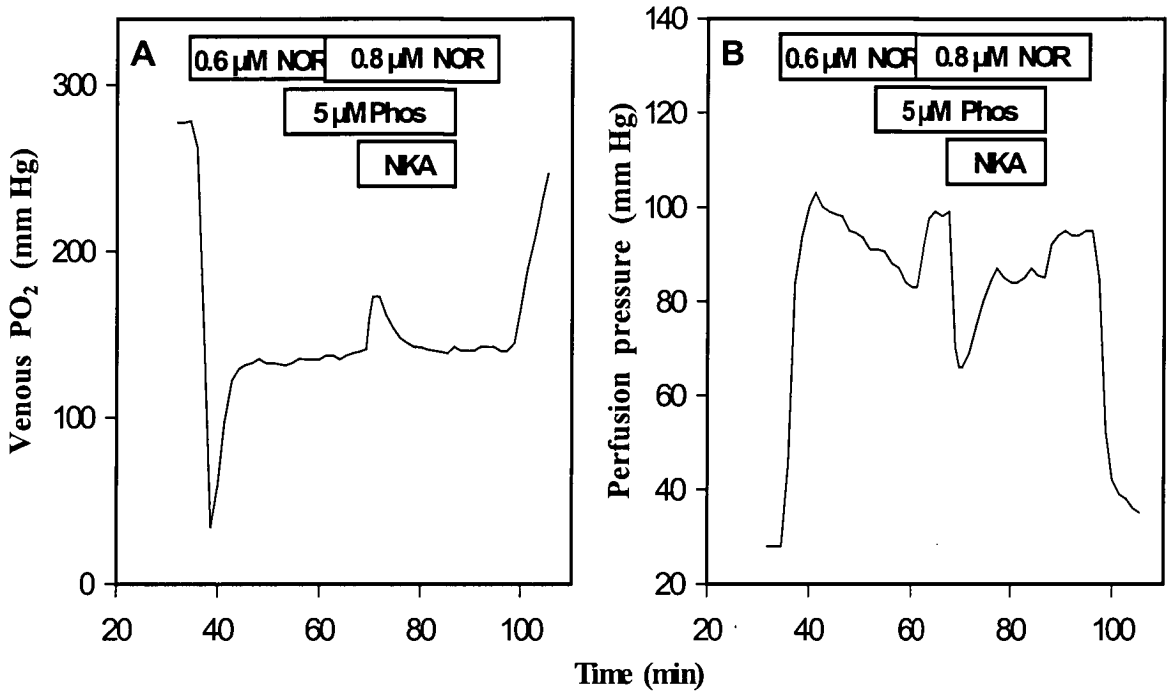


Fig. 4.6. Typical tracing of the peak and steady state actions of 20 nM neurokinin A (NKA) on venous partial pressure of oxygen (PO_2) (panel A) and arterial perfusion pressure (panel B), under noradrenalin (NOR) stimulation, in the perfused rat hindlimb.

the reduction in venous PO_2 (stimulation of VO_2) by NOR (Fig. 4.6A), and produced a strong peak vasodilator response that, unlike SP, eventually reached steady state at a perfusion pressure below that seen with 0.8 µM NOR and phosphoramidon alone (Fig. 4.7B). The removal of NKA and phosphoramidon, but not NOR, resulted in a rapid increase in perfusion pressure, while venous PO_2 remained relatively constant.

4.3.4.2. NKA-induced effects and the importance of nitric oxide formation

Repeat experiments examining the effects of NKA in NOR-stimulated perfused hindlimb confirmed the preliminary experiment described above (Fig. 4.7A, C). Once again, the infusion of 0.6 µM NOR stimulated hindlimb VO_2 (ΔVO_2, 3.53 ± 0.11 µmol. $g.^{-1}$ h^{-1}) and increased arterial perfusion pressure (ΔPP, 68.0 ± 5.2 mm Hg), with the pressure, but not VO_2, being reduced by the subsequent infusion of phosphoramidon (ΔVO_2, 3.53 ± 0.16 µmol. $g.^{-1}$ h^{-1}; ΔPP, 56.5 ± 5.1 mm Hg). These data are not shown in Fig 4.7 due to alterations in the protocol (using L-NAME). Perfusion pressure was

again restored by increasing the NOR concentration (0.8 μM NOR: ΔVO_2, 3.48 ± 0.13 μmol. $g.^{-1}$ h^{-1}; ΔPP, 70.5 ± 7.0 mm Hg, Fig. 4.7). A low concentration of NKA (20 nM) partly reversed the NOR-induced stimulation of VO_2 (peak ΔVO_2, 2.73 ± 0.14 μmol. $g.^{-1}$ h^{-1}, Fig. 4.7A), and induced a strong, statistically significant ($P<0.05$, Table 4.5) peak vasodilatation (peak ΔPP, 39.3 ± 5.3 mm Hg, Fig. 4.7C). However, under continued NKA infusion, the VO_2 response returned to previous NOR-stimulated levels (ΔVO_2, 3.33 ± 0.14 μmol. $g.^{-1}$ h^{-1}) and perfusion pressure partly recovered to, though still remained below, the previous NOR-stimulated pressure (ΔPP, 59.5 ± 5.0 mm Hg, Fig 4.7C).

The experiments with NKA in the presence of L-NAME were simplified by altering the infusion protocol to begin with the infusion of 0.8 μM NOR and 5 μM phosphoramidon, eliminating the initial concentration of NOR (0.6 μM) (Fig. 4.7B, D). This maximised the use of the peptidase inhibitor for its intended purpose, to preserve NKA during its infusion. The improved protocol was not adopted in previous experiments as it was necessary to confirm the vasodilatory effect of phosphoramidon, given the ambiguity of the response to this inhibitor in the experiments with CGRP and L-NAME. The minor alteration to the protocol yielded very similar VO_2 and perfusion pressure responses to 0.8 μM NOR, in the presence of phosphoramidon, to those seen in the previous experiments without L-NAME (ΔVO_2, 3.35 ± 0.16 μmol. $g.^{-1}$ h^{-1}; ΔPP, 71.8 ± 5.6 mm Hg). The infusion of L-NAME (10 μM) did not cause any apparent alteration in these VO_2 and pressure values (ΔVO_2, 3.35 ± 0.16 μmol. $g.^{-1}$ h^{-1}; ΔPP, 72.5 ± 5.8 mm Hg, $P>0.05$, Table 4.5). Unlike the data in the absence of L-NAME (Fig. 4.7A), the infusion of NKA into the preparation in the presence of L-NAME did not induce a peak reversal of the stimulation of VO_2 by NOR (ΔVO_2, 3.18 ± 0.27 μmol. $g.^{-1}$ h^{-1}), nor was there an observed change in VO_2 by NKA under steady state conditions (ΔVO_2, 3.38 ± 0.17 μmol. $g.^{-1}$ h^{-1}, Fig. 4.7B). This was confirmed by the lack of statistical significance in both repeated measures ANOVA, and subsequent post-hoc tests of the data (Table 4.5).

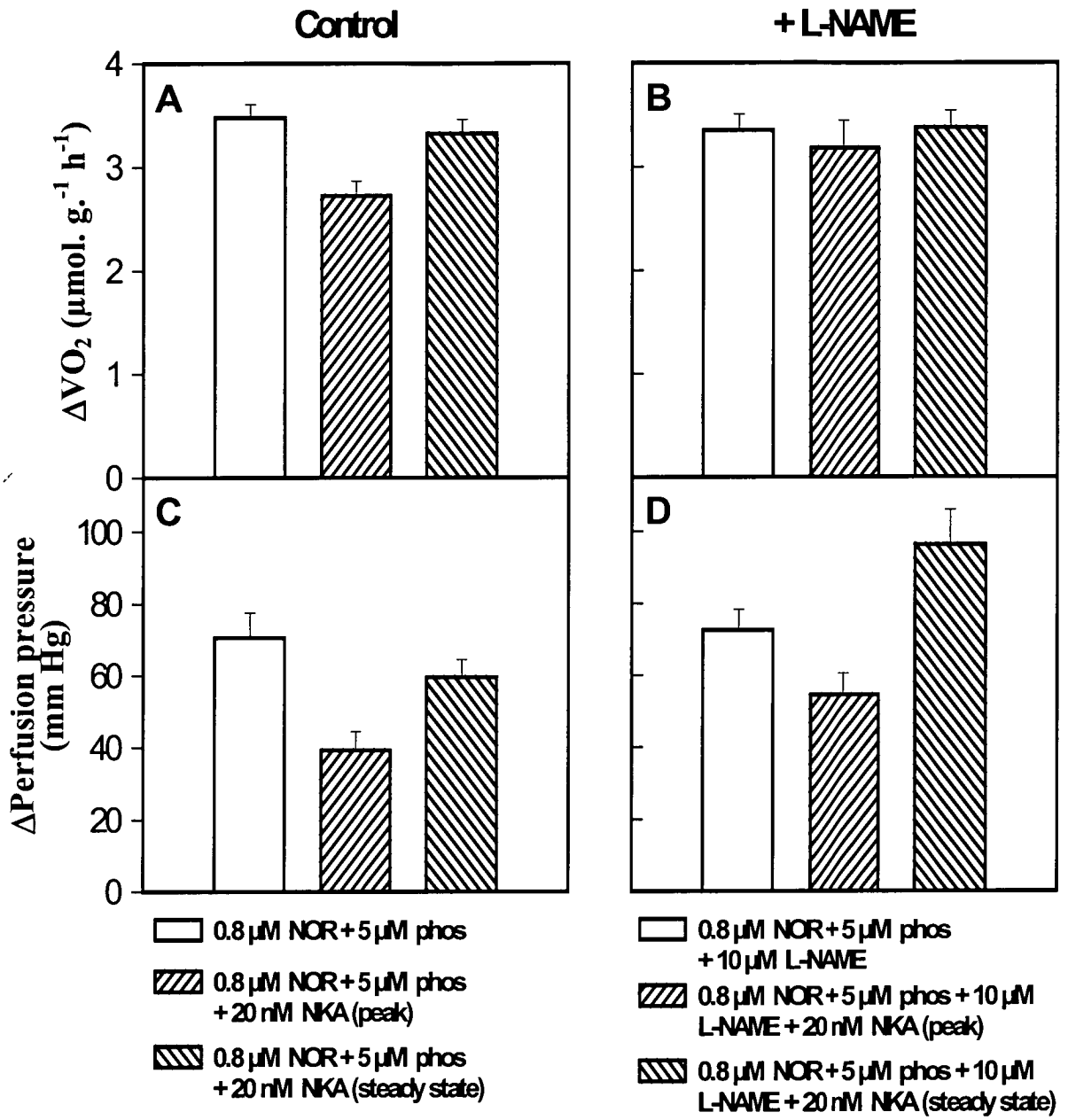


Fig. 4.7. Effect of neurokinin A (NKA) and phosphoramidon (phos) on noradrenalin (NOR)-induced oxygen consumption (top panels) and perfusion pressure (bottom panels) changes in the perfused rat hindlimb (n = 4). Responses to NKA were examined in the absence (panels A and C) and presence (panels B and D) of the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). Statistical analysis of the data by one-way repeated measures ANOVA and pairwise comparisons (Student-Newman-Keuls method) is presented in Table 4.5.

TABLE 4.5. One-way repeated measures analysis of variance (ANOVA) and pairwise multiple comparisons (Student-Newman-Keuls method) of the data presented in Fig. 4.7 for NKA-induced changes in oxygen consumption (ΔVO_2), and perfusion pressure (ΔPP) in the absence and presence of L-NAME.

Test	Figure	ΔVO_2	ΔPP
[1]ANOVA P value	4.7A, C	<0.001	<0.001
F statistic	4.7A, C	69.30	61.57
[1]ANOVA P value	4.7B, D	0.247	<0.001
F statistic	4.7B, D	1.56	16.50
[2]S-N-K comparisons:			
[3]NOR + phos vs. NKA (peak) + NOR + phos	4.7A, C	*	*
[3]NOR + phos vs. NKA (steady state) + NOR + phos	4.7A, C	ns	*
[3]NOR + phos vs. L-NAME + NOR + phos	4.7B, D	ns	ns
[3]NOR + phos + L-NAME vs. NKA (peak) + NOR + phos + L-NAME	4.7B, D	ns	*
[3]NOR + phos + L-NAME vs. NKA (steady state) + NOR + phos + L-NAME	4.7B, D	ns	*

[1]ANOVA P value significant at P<0.05

[2]Student-Newman-Keuls P value significant at P<0.05, * = significant, ns = not significant.

[3]NOR concentration = 0.8 μM

Conversely, NKA did cause a peak dilatation in the presence of L-NAME (ΔPP, 54.5 ± 5.98 mm Hg, Fig., 4.7D) that achieved statistical significance ($P<0.05$, Table 4.5). However, this reduction in NOR-induced pressure was calculated to be 42% less than the peak NKA-induced dilatation in the absence of L-NAME. In addition, steady state perfusion pressure in the presence of NOR, NKA and L-NAME was significantly ($P<0.05$, Table 4.5) greater than that induced by NOR in the absence of the peptide (ΔPP, 96.3 ± 9.7 mm Hg, Fig. 4.7D). Hence in the presence of the NOS inhibitor, NKA caused steady state vasoconstriction, rather than dilatation.

4.3.5. Carbachol-induced effects in noradrenalin-stimulated perfused hindlimb

4.3.5.1. Time course for the actions of carbachol

The actions of the NO-dependent vasodilator CCh (0.5 μM) in a perfused hindlimb preparation stimulated with NOR (0.6 μM) are presented in Figure 4.8. Under NOR-induced changes in hindlimb venous PO_2 and vascular tension, the infusion of CCh into the preparation caused a partly sustained reversal of venous PO_2 (Fig. 4.8A), and strong, fully sustained dilatation of the hindlimb vasculature (Fig. 4.8B). Removal of both NOR and CCh resulted in the return of venous PO_2 and perfusion pressure to their previous

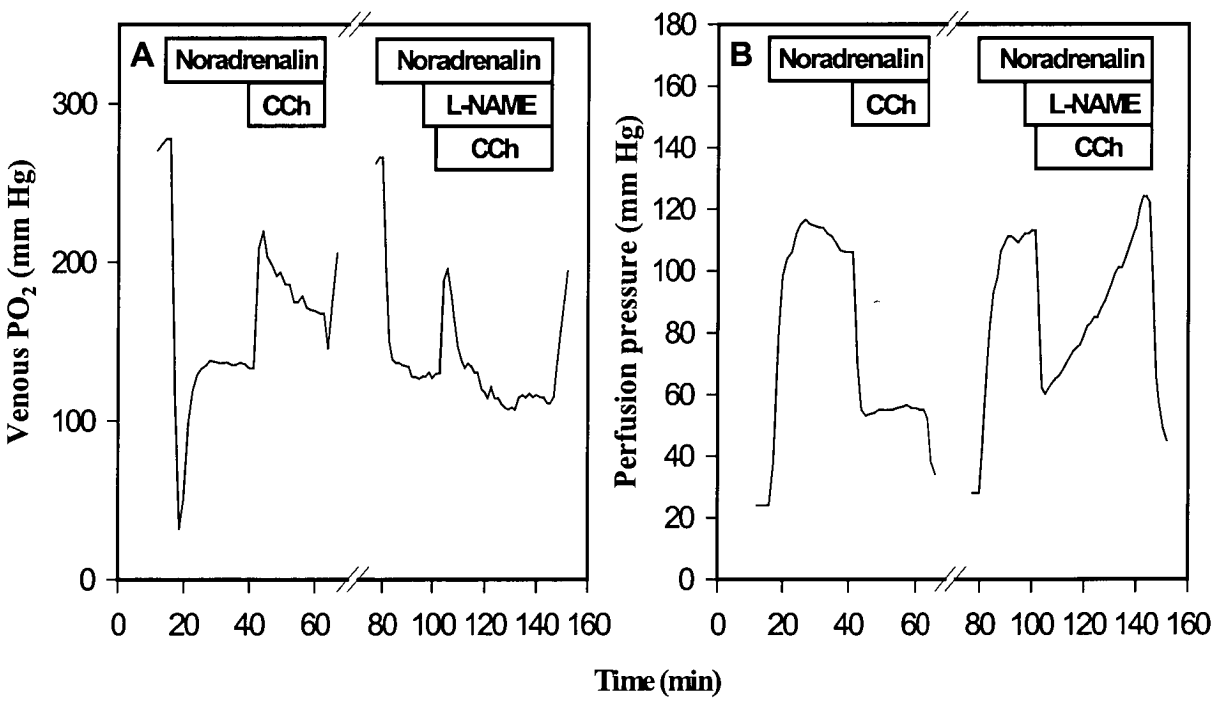


Fig. 4.8. Typical tracing of the actions of 0.5 μM carbachol (CCh) on venous partial pressure of oxygen (PO_2) (panel A) and arterial perfusion pressure (panel B), under noradrenalin (NOR) stimulation, in the perfused rat hindlimb. In the same perfusion, the sensitivity of CCh vascular and metabolic actions to the nitric oxide synthase inhibitor L-NAME (10 μM) was examined.

unstimulated (basal) steady state levels. The protocol of NOR and CCh infusion was then repeated in the presence of L-NAME (10 μM) to inhibit NO formation. The repeat infusion of NOR caused an almost identical steady state decrease in venous PO_2 and

increase in perfusion pressure. The absence of a peak decrease in venous PO_2 during the second NOR infusion is probably due to the lack of erythrocyte wash-out from previously underperfused regions of the muscle, seen only in the initial NOR infusion. The temporary presence of erythrocytes in the venous outflow would undoubtedly cause a transient decrease the amount of oxygen dissolved, and therefore detected, in the extracellular perfusate. The co-infusion of L-NAME with NOR into the preparation did not produce detectable changes in either venous PO_2 or perfusion pressure. The addition of CCh in the presence of NOR and L-NAME caused distinctly different changes in venous PO_2 and pressure to those seen in the first infusion without L-NAME present. CCh stimulated a transient reversal of the NOR-induced decrease in venous PO_2 that, unlike its effect in the absence of L-NAME, reached a steady state value below that seen with NOR alone. Similarly, peak dilatation by CCh was still evident in the presence of L-NAME, however this dilatation was not sustained and perfusion pressure gradually increased to a level above that induced by NOR alone, resulting in increased vascular tone.

4.3.5.2. Sensitivity of carbachol-induced dilatation to L-NAME

Figure 4.9 shows the results of several experiments using a protocol identical to that seen in Figure 4.8. As for previous experiments, NOR (0.6 μM) stimulated hindlimb VO_2 (ΔVO_2, 3.38 ± 0.09 μmol. $g.^{-1}$ h^{-1}, Fig. 4.8A) and markedly increased perfusion pressure (ΔPP, 89.3 ± 2.8 mm Hg, Fig. 4.8B). The infusion of CCh (0.5 μM) elicited a peak reversal of NOR-induced VO_2 (ΔVO_2, 1.85 ± 0.15 μmol. $g.^{-1}$ h^{-1}) that eventually reached steady state (ΔVO_2, 2.9 ± 0.16 μmol. $g.^{-1}$ h^{-1}), both of which were statistically significant from NOR-induced VO_2 change ($P<0.05$, Table 4.6). CCh also stimulated a sustained, statistically significant ($P<0.05$, Table 4.6) decrease in hindlimb vasculature tension (i.e. dilatation) (ΔPP, 34.0 ± 2.0 mm Hg) compared to the increased pressure during NOR infusion.

After the return to steady state basal VO_2 and perfusion pressure, a repeat infusion of NOR stimulated VO_2 and increased perfusion pressure in an almost identical manner to the first NOR infusion (ΔVO_2, 3.45 ± 0.07 μmol. $g.^{-1}$ h^{-1}; ΔPP, 92.0 ± 5.7 mm Hg). The infusion of L-NAME (10 μM) did not significantly ($P>0.05$, Table 4.6) alter any of

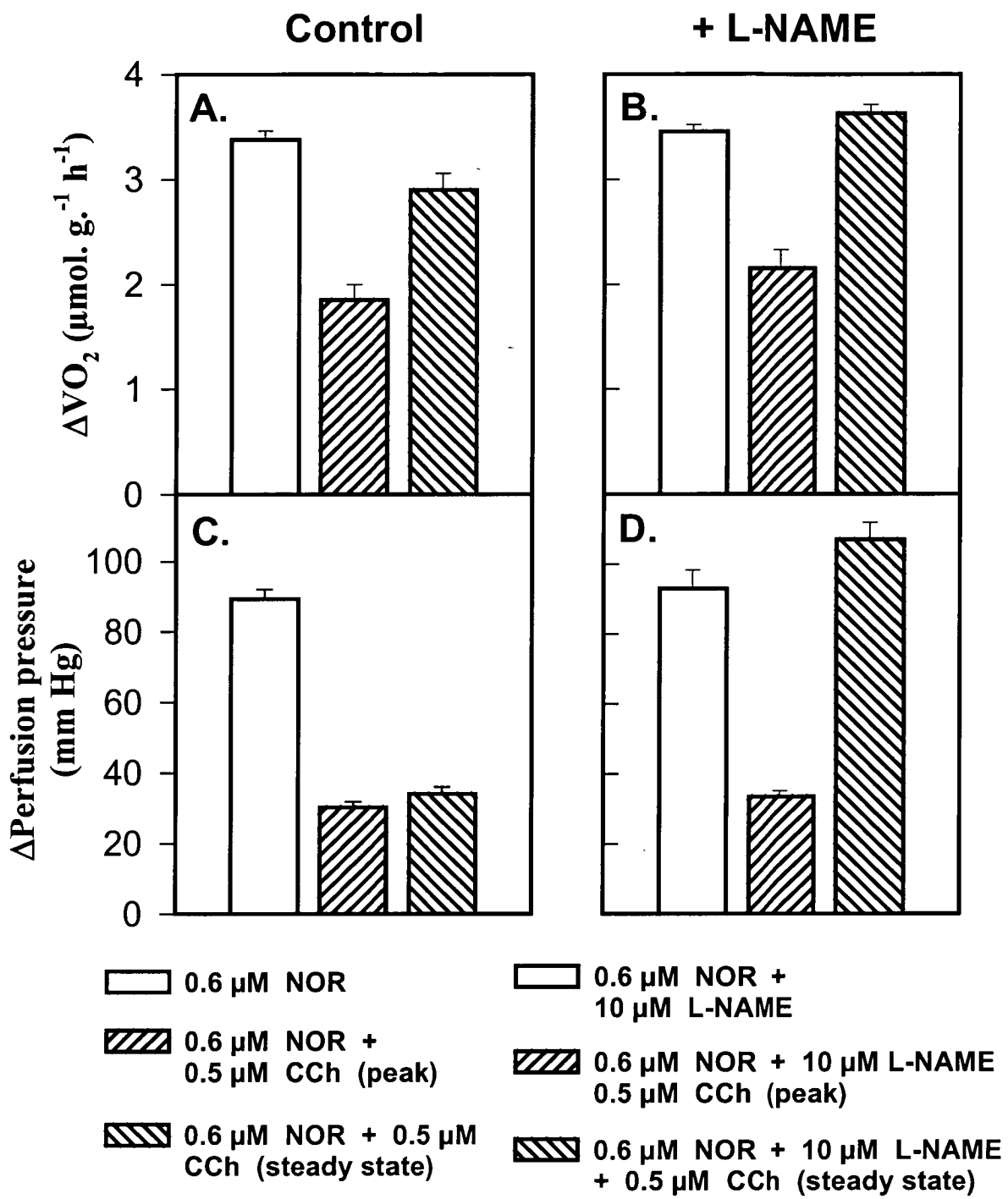


Fig. 4.9. Effect of carbamyl choline (carbachol, CCh) on noradrenalin (NOR)-induced oxygen consumption (panel A) and perfusion pressure (panel B) changes in the perfused rat hindlimb (n = 4). Statistical analysis of the data by one-way repeated measures ANOVA and pairwise comparisons (Student-Newman-Keuls method) is presented in Table 4.6.

TABLE 4.6. One-way repeated measures analysis of variance (ANOVA) and pairwise multiple comparisons (Student-Newman-Keuls method) of the data presented in Fig. 4.9 for carbachol-induced changes in oxygen consumption (ΔVO_2), and perfusion pressure (ΔPP) in the absence and presence of L-NAME.

Test	Figure	ΔVO_2	ΔPP
[1]ANOVA P value	4.9	<0.001	<0.001
F statistic	4.9	48.93	201.44
[2]S-N-K comparisons:			
NOR vs. NOR + CCh (peak)	4.9	*	*
NOR vs. NOR + CCh (steady state)	4.9	*	*
[3]NOR vs. L-NAME + NOR	4.9	ns	ns
[3]NOR + L-NAME vs. [4]CCh (peak) + NOR + L-NAME	4.9	*	*
[3]NOR + L-NAME vs. [4]CCh (45 min) + NOR + L-NAME	4.9	*	*

[1]ANOVA P value significant at P<0.05

[2]Student-Newman-Keuls P value significant at P<0.05, * = significant, ns = not significant

[3]Repeat NOR infusion

[4]Repeat CCh infusion

these parameters (ΔVO_2, 3.45 ± 0.07 μmol. $g.^{-1}$ h^{-1}; ΔPP, 93.0 ± 5.2 mm Hg). The repeat infusion of CCh (0.5 μM) elicited a significant (P<0.05, Table 4.6) peak reversal of NOR-induced VO_2 and vascular effects (ΔVO_2, 2.15 ± 0.18 μmol. $g.^{-1}$ h^{-1}; ΔPP, 33.5 ± 1.7 mm Hg). Contrary to the data obtained in the absence of L-NAME, these effects of CCh in the presence of the NOS inhibitor were not sustained. Indeed the steady state VO_2 change under CCh-stimulation in these circumstances was similar to that induced by NOR alone (Fig. 4.9A). Conversely, perfusion pressure under CCh stimulation in the presence of L-NAME did not reach steady state with up to 45 minutes infusion time, and gradually increased over this period. Perfusion pressure readings taken at 45 minutes, however, were significantly (P<0.05, Table 4.6) greater than those stimulated by NOR alone (ΔPP, 107.0 ± 4.9 mm Hg, Fig. 4.9B).

4.3.6. Effect of capsaicin in noradrenalin-stimulated perfused hindlimb

4.3.6.1. Time course for the actions of capsaicin

In the perfused rat hindlimb preparation under NOR-induced vascular tension, and decreased venous PO_2, a low concentration of capsaicin (50 nM) caused a mild increase in perfusion pressure (vasoconstriction), but had no apparent effect on venous PO_2 (Fig. 4.10). A ten-fold higher concentration of capsaicin (0.5 μM) induced a rapid, strong vasoconstriction that peaked and then returned slowly to the perfusion pressure observed prior to vanilloid infusion. In addition, there was a sustained reversal of NOR-induced venous PO_2 change at this concentration of capsaicin. Interestingly, the

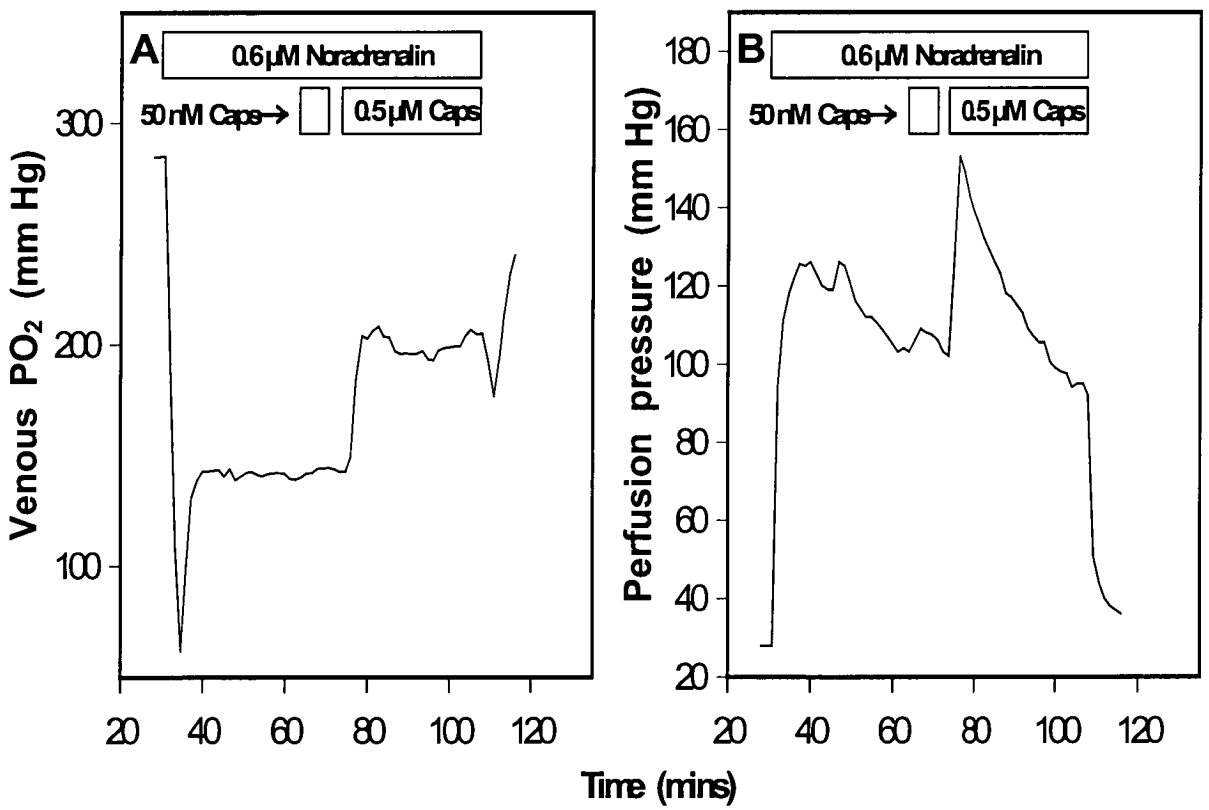


Fig. 4.10. Typical tracing of the actions of 50 nM and 0.5 μM capsaicin (Caps) on venous partial pressure of oxygen (PO_2) (panel A) and arterial perfusion pressure (panel B), under noradrenalin stimulation, in the perfused rat hindlimb.

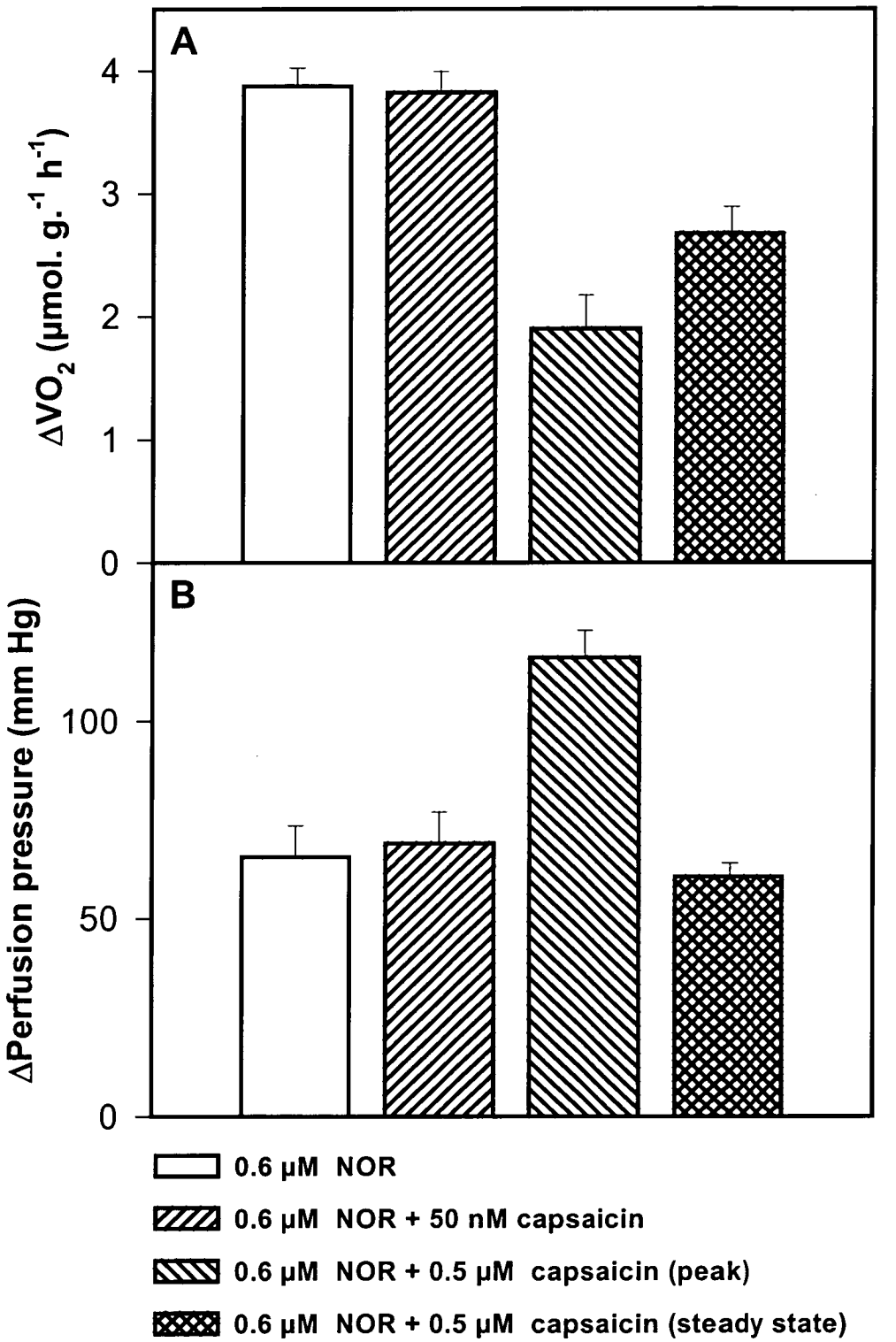


Fig. 4.11. Effect of capsaicin on noradrenalin (NOR)-induced oxygen consumption (panel A) and perfusion pressure (panel B) changes in the perfused rat hindlimb (n = 4). Statistical analysis of the data by one-way repeated measures ANOVA and pairwise comparisons (Student-Newman-Keuls method) is presented in Table 4.7.

removal of both NOR and capsaicin resulted in a peak decrease in venous PO_2, before returning to a basal (unstimulated) level.

4.3.6.2. Capsaicin-induced vasoconstriction under noradrenalin-induced vascular tension

The actions of capsaicin just described (Fig. 4.10) were confirmed in further experiments, the results of which are shown in Figure 4.11. NOR (0.6μM) stimulated a characteristic increase in hindlimb VO_2 (ΔVO_2, 3.88 ± 0.15 μmol. $g.^{-1}$ h^{-1}, Fig. 4.11A) and raised perfusion pressure well above that seen under basal conditions (ΔPP, 65.5 ± 8.1 mm Hg, Fig 4.11B). The co-infusion of 50 nM capsaicin with NOR did not

TABLE 4.7. One-way repeated measures analysis of variance (ANOVA) and pairwise multiple comparisons (Student-Newman-Keuls method) of the data presented in Fig. 4.11 for capsaicin-induced changes in oxygen consumption (ΔVO_2), and perfusion pressure (ΔPP).

Test	Figure	ΔVO_2	ΔPP
[1]ANOVA P value	4.11	<0.001	<0.001
F statistic	4.11	20.82	124.53
[2]S-N-K comparisons:			
NOR vs. 50 nM capsaicin	4.11	ns	ns
NOR vs. 0.5 μM capsaicin (peak) + NOR	4.11	*	*
NOR vs. 0.5 μM capsaicin (steady state) + NOR	4.11	*	ns

[1]ANOVA P value significant at P<0.05

[2]Student-Newman-Keuls P value significant at P<0.05, * = significant, ns = not significant

significantly (P>0.05, Table 4.7) alter VO_2 or perfusion pressure (ΔVO_2, 3.83 ± 0.07 μmol. $g.^{-1}$ h^{-1}; ΔPP, 69.0 ± 8.0 mm Hg), although a mild perfusion pressure increase, similar to that shown in Figure 4.10, was consistently observed. A higher concentration of capsaicin (0.5 μM) elicited a significant (P<0.05, Table 4.7) peak reversal of NOR-induced VO_2 change (ΔVO_2, 1.90 ± 0.27 μmol. $g.^{-1}$ h^{-1}), and further increased perfusion pressure (ΔPP, 116.0 ± 6.9 mm Hg).

While the effect of 0.5 μM capsaicin on VO_2 was partly sustained (ΔVO_2, 2.68 ± 0.22 μmol. $g.^{-1}$ h^{-1}), its effect on perfusion pressure was not and gradually attained a steady state value that was not statistically significant ($P>0.05$, Table 4.7) from that induced by NOR alone (ΔPP, 60.5 ± 3.5 mm Hg).

4.4 Discussion

4.4.1. Calcitonin gene-related peptide-induced vasodilatation and NO-dependence

In the perfused rat hindlimb under NOR-induced vascular tension, a low concentration of CGRP induced a strong, sustained dilatation that was evident as a 54% decrease in hindlimb perfusion pressure (vascular resistance). CGRP caused comparatively weak dilatation (20% decrease in vascular resistance) in the perfused hindlimb stimulated with the vasoconstrictor 5-HT, although this dilatation was also sustained. However, there is now strong evidence to suggest that NOR and 5-HT, although both vasoconstrictors, cause opposite changes in the distribution of perfusate flow in the rat hindlimb that is reflected in their opposite effect on hindlimb VO_2 (reviewed in Clark *et al.*, 1995; 1997). Indeed under constant-flow conditions, 5-HT has been shown to increase non-nutritive flow in the perfused hindlimb by redistributing perfusate to connective tissue vessels, reducing flow to nutritive vessels within muscle (Newman *et al.*, 1997). Consequently, CGRP probably has limited access to its receptors under 5-HT stimulation, whereas access is likely to be greater during the increased nutritive flow seen under stimulation by NOR, and may explain the apparent increase in CGRP efficacy during catecholamine stimulation. Similar flow-dependent changes in efficacy have been observed in this preparation by infusing a type A vasoconstrictor (AII) under conditions of predominantly non-nutritive flow induced by 5-HT (Newman *et al.*, 1998). When 5-HT is infused prior to AII, the thermogenic and vasoconstrictor effects of the latter are greatly reduced compared to the infusion of AII alone. On the other hand, if AII is infused prior to 5-HT, the vasoconstriction and decreased VO_2 by the latter are enhanced when compared to the infusion of 5-HT alone. This probably reflects an increase in nutritive flow by AII and, therefore, greater access of 5-HT to its receptors.

In the studies presented in this chapter, CGRP-induced dilatation was shown to be independent of NO formation given the inability of the NOS inhibitor L-NAME to

block the effects of the neuropeptide. Conversely, the same concentration of L-NAME (10 μM) was effective in blocking the sustained vasodilatation and VO_2 effects induced by the ACh mimetic CCh. In fact, in the presence of L-NAME, CCh caused a slow-onset vasoconstrictor effect in the perfused hindlimb. The inability of L-NAME to block the peak vasodilator response to CCh was not unexpected since ACh, the endogenous analogue of this dilator, has L-NAME-sensitive and an L-NAME-insensitive components to its vasorelaxing effect (Doyle and Duling, 1997).

Similar to the findings presented in this chapter for CGRP are those of Kim *et al.* (1995) who showed that CGRP- and capsaicin-induced arteriolar dilatation in rat striated muscle (cremaster) were not inhibited by a high concentration of L-NAME (10^{-4} M), however venous dilatation by the peptide was sensitive to the NOS inhibitor. In addition, exercise-induced active hyperaemia in the rat hindlimb is sensitive to $CGRP_{(8-37)}$, but not to L-NAME suggesting the presence of NO-independent dilatation mediated by the release of endogenous CGRP (Yamada *et al.*, 1997a; 1997b). On the other hand, studies by Didion and Mayhan (1997) in rats with impaired arterial reactivity, due to chronic myocardial infarction, suggest that arteriolar dilatation by CGRP in skeletal muscle is dependent on the formation of NO. In myocardial-infarcted rats, ACh- and CGRP-induced (but not SP- or nitroprusside-induced) dilatation in the rat spinotrapezius muscle is reduced when compared to control rats, but is partly restored by the suffusion of L-arginine, an essential substrate for the formation of NO by NOS. Hence there is ambiguity in the involvement of NO in CGRP-induced dilatation in skeletal muscle, an observation that is not unusual since it is well known that CGRP employs both NO-dependent and NO-independent signal transduction mechanisms to stimulate vasorelaxation (reviewed by Bell and McDermott, 1996). An excellent example of the heterogeneity of CGRP-NO interactions is evident in two different tissue types where CGRP is the major mediator of afferent nerve mediated vasodilatation - namely the skin and gastric mucosa of the rat (Holzer *et al.*, 1995). It has been demonstrated in the stomach microcirculation that CGRP-induced vasodilatation occurs by the stimulation of endothelial CGRP receptors and synthesis and release of NO, although an NO-independent mechanism is observed at higher CGRP concentrations (Holzer *et al.*, 1993). However, in the cutaneous microcirculation in the rat, CGRP stimulates vasodilatation by a direct mechanism that is not blocked by L-NAME and therefore

does not involve NO synthesis (Ralevic *et al.*, 1992; Holzer and Jocic, 1994), but instead stimulates vascular smooth cell cAMP production (Marshall, 1992).

4.4.2. Tachykinin-induced vasodilatation and NO-dependence

Vascular responses to the tachykinins SP and NKA in the perfused hindlimb under NOR-induced vascular tension were quantitatively and qualitatively similar. Both peptides induced a strong peak dilatation that was partly sustained with NKA, but fully reversed under continuous SP infusion. The reversal of the NOR-induced increase in VO_2 by both peptides was also transient and was not sustained for the duration of peptide infusion. A similar vascular effect by SP has been observed in the rat cremaster muscle preparation where the peptide stimulates strong but brief dilatation of small resistance vessels, lasting less than 5 minutes (Brock and Joshua, 1991). Repeated exposure to a picomolar concentration of SP in the rat cremaster, separated by 20 minute recovery periods, still caused significant dilatation, however continuous exposure to SP substantially decreased the magnitude of dilatation. A similar tachyphylactic response to SP was observed in an earlier study by Lembeck and Holzer (1979) where the increased flow in the rat hindpaw following SP infusion into the femoral artery eventually disappeared under continuous peptide infusion. Hence in the data presented in this chapter, the transient nature of tachykinin-induced dilatation may be due to rapid desensitisation (tachyphylaxis), a frequently observed phenomenon that is likely to be due to the internalisation of tachykinin receptors (Bowden *et al.*, 1994). Alternatively, SP or NKA may stimulate the release of an endogenous vasoconstrictor in a similar manner to that seen in the isolated rabbit intrapulmonary artery where SP stimulates vasoconstriction by the secondary release of thromboxane A_2 (Shirahase *et al.*, 1995). If such a mechanism occurs in muscle then reversal (either partly or fully) of the initial vasodilator effect of the tachykinins would be expected.

In the studies presented in this chapter, it appears that the vascular actions of the tachykinins in the NOR-stimulated perfused rat hindlimb are partly mediated by the formation of NO by NOS, particularly with respect to NKA. Both the peak and steady state vasodilatation induced by NKA were sensitive to the NOS inhibitor L-NAME, however the former was not completely abolished. Nonetheless, NKA caused steady state vasoconstriction, rather than dilatation, in the presence of L-NAME and it is likely that NO plays a significant role in the vascular response to this peptide. Although the

initial peak vasodilator response to SP was not altered by L-NAME, steady state vascular tone in the presence of SP + L-NAME was consistently higher than that prior to peptide infusion. Thus, under NOS inhibition SP is a steady state vasoconstrictor and it appears that NO may play a role in the maintenance of vascular tone, but not the initial transient dilatation by this peptide. The mechanisms underlying the peak dilatation by SP in this preparation are unclear at present, but may involve the release of other factors such as endothelium-derived hyperpolarising factor (EDHF). Indeed, similar findings have been observed by others where SP induced a biphasic vasorelaxation, with the initial peak dilatation being insensitive to NOS inhibition, but the steady state response being completely ablated (Kuroiwa *et al.*, 1995). Furthermore, these authors showed that eliminating the effects of EDHF (with 40 mM K^+-depolarisation) also abolished the initial peak dilatation caused by SP.

While it is widely accepted that tachykinin-induced vasodilatation, particularly by SP, is mediated by the stimulation of endothelial NK1 receptors (reviewed by Maggi *et al.*, 1993b; Lundberg, 1996) there is ambiguity with regard to the role of NO formation in these vasodepressor responses. Vasodilatation by tachykinins is blocked by NOS inhibition in tissues from the rat (Whittle *et al.*, 1989; Brock and Joshua, 1991; Huang and Koller, 1996), hamster (Hall and Brain, 1994), pig (Kuroiwa *et al.*, 1995) and rabbit (Persson *et al.*, 1991; Gustafsson *et al.*, 1994). However, in other preparations, SP can evoke vasodilatation under NOS inhibition (Kerezoudis *et al.*, 1993; Shiramoto *et al.*, 1997), and its vasodilator action is not affected by vascular dysfunction in rat spinotrapezius muscle arterioles, unlike some NO-dependent dilators (Didion and Mayan, 1997).

Recent studies in the human forearm have highlighted an interesting example of the uncertainty surrounding NO involvement in tachykinin-mediated vasodilatation. Shiramoto and his co-workers have shown similar increases in forearm blood flow in response to ACh, ATP sodium nitroprusside (SNP) and SP, however only the dilatation induced by ACh was sensitive to the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) (Shiramoto *et al.*, 1997). In addition, the infusion of L-arginine, the substrate for NOS, did not augment the vasodilator response to SP. Furthermore, the same group have published data showing that in patients with endothelial dysfunction due to heart failure, the vasodilator response to ACh, but not SP or SNP, was diminished and was restored by infusion of L-arginine (Hirooka *et al.*, 1992).

In contrast to the studies of Shiramoto *et al.* (1997), Newby *et al.* (1997a) have published data using the same preparation but drew more or less opposite conclusions on the role of NO in SP-induced vasodilatation. The two studies showed strong similarity in both resting and ACh-stimulated forearm blood flow, however the latter study showed a markedly reduced response to SP that showed greater sensitivity to L-NMMA than did ACh. These findings are consistent with other studies in the human forearm where L-NMMA was effective at blocking the vasodilator response to SP (Cockcroft *et al.*, 1994; Panza *et al.*, 1994), but did not further reduce vasodilator responses to SP in patients with endothelial dysfunction due to hypertension (Panza *et al.*, 1994). However, in the studies of Newby *et al.* (1997a) and Cockcroft *et al.* (1994) the observed dilator response to SP was markedly lower than that seen by Shiramoto's group, who showed some reduction in SP-induced vasodilatation by L-NMMA that did not achieve statistical significance. On the other hand, Shiromoto *et al.* did not infuse L-NMMA continuously in their study, although Panza *et al.* followed the same protocol and were able to block vasodilator responses to SP that were similar in magnitude. Thus, the apparent discrepancies with regard to NO involvement in SP-induced vasodilatation in this preparation are not likely to be due to differences in the experimental protocol adopted by the various study groups, but may instead be due to the source of SP used (Wallace, 1997) or racial differences (Shiramoto and Imaizumi, 1997).

4.4.3. Capsaicin-induced vasoconstriction under NOR-stimulated vascular tone

Despite capsaicin's well-documented ability to stimulate the release of sensory neuropeptides (reviewed by Holzer, 1991; Maggi, 1993) and the demonstrated ability of the sensory peptides to induce vasodilatation in the present studies, the infusion of capsaicin into the hindlimb preparation under NOR-induced vascular tone elicited strong vasoconstriction. However, the vasoconstriction induced by capsaicin was not sustained and perfusion pressure slowly returned to that induced by NOR alone. Why the vasoconstrictor response was transient is unclear since similar concentrations of capsaicin cause sustained vasoconstriction in the unstimulated perfused hindlimb preparation (Cameron-Smith *et al.* 1990; Colquhoun *et al.*, 1995; Chapter 1, Fig. 1.2). However, under NOR-induced vasoconstriction, this concentration of capsaicin was markedly more potent and may have elicited rapid desensitisation. Furthermore, under

stimulation by NOR, the perfusion pressure in these studies prior to capsaicin infusion was much greater than the maximum capsaicin-induced perfusion pressure response normally seen in the unstimulated perfused hindlimb.

4.4.4. Capsaicin and neuropeptide effects on VO_2 in the vasoconstrictor-stimulated perfused hindlimb

A continuing pattern to emerge from the studies presented in this chapter was that NOR-induced increases in VO_2 in the constant-flow perfused rat hindlimb were reversed by the subsequent infusion of either vasodilator (CCh, CGRP, SP, NKA) or vasoconstrictor (capsaicin) agents. Thus, at the concentrations used in this study, NOR-induced stimulation of VO_2 probably represents near-maximal nutritive vascular flow, particularly since in all experiments increasing the concentration of NOR from 0.6 μM to 0.8 μM did not further increase VO_2 but, in some cases, partly reversed VO_2 stimulation. Hence any alteration of this nutritive/non-nutritive flow distribution, either by peptide- or CCh-induced dilatation, or capsaicin-induced vasoconstriction, results in the reversal of NOR-induced VO_2 effects. Conversely, under conditions of predominantly non-nutritive flow presumed to occur with the infusion of 5-HT, a change in vascular tension by the infusion of CGRP resulted in an increase in hindlimb VO_2, and probably reflects a redistribution of perfusate flow to nutritive vascular beds.

Whether or not the flow redistribution by stimulatory concentrations of NOR is indicative of that which occurs *in vivo*, under basal conditions, is unclear at present. Indeed, it seems more likely that perfused hindlimb flow distribution under type A concentrations of NOR (i.e. those which stimulate VO_2) more closely resembles that which occurs during maximal exercise since type A vasoconstrictors are able to improve hindlimb muscle contraction (Dora *et al.*, 1994; Rattigan *et al.*, 1996). Consequently, from the experiments presented in this chapter, it is difficult to draw any firm conclusions about the likely actions of sensory neuropeptides or capsaicin on skeletal muscle VO_2 *in vivo* since their effects on muscle metabolism in the perfused hindlimb may be masked by the strong thermogenic effect of NOR. Their actions on the hindlimb vasculature in these studies, however, were more conclusive since arterial pressure under NOR-stimulation more closely resembles *in vivo* arterial pressure.

Chapter 5

General Discussion and Conclusions

5.1. Summary and Discussion of Major Findings

5.1.1. Further evidence for vanilloid receptor subtypes in the perfused rat hindlimb

Capsaicin-induced vasoconstriction in isolated arteries has been attributed in the past to non-selective effects that occur via the direct physicochemical interaction of capsaicin with smooth muscle plasma membranes, and do not exhibit desensitisation (reviewed by Maggi and Meli, 1988; Holzer, 1991). While the repeated infusion of vanilloids in the rat hindlimb does not produce acute desensitisation (Eldershaw *et al.*, 1994), a hallmark of the specific capsaicin action on sensory neurons, the findings presented in Chapter 2 clearly indicate that the effects produced by capsaicin in this preparation are highly selective and very likely to be receptor-mediated. Moreover, using specific competitive and non-competitive vanilloid receptor antagonists, further evidence has been obtained in support of the hypothesis proposed by Colquhoun *et al.* (1995) that vanilloid receptor subtypes (VN_1 and VN_2 receptors) control the biphasic VO_2 responses to capsaicin in the perfused rat hindlimb. A low concentration (100 nM) of the non-competitive antagonist ruthenium red was used to selectively block the VO_2 and vasoconstrictor effects caused by the infusion of high capsaicin concentrations into this preparation (Chapter 2, Fig. 2.5). These findings may be indicative of capsaicin cation channel heterogeneity where the putative VN_2 receptor/channel complex is more sensitive to ruthenium red blockade. Low concentrations of capsazepine were used to selectively block the stimulation of VO_2 by capsaicin, but also potentiated the inhibition of VO_2 by the vanilloid (Chapter 2, Fig. 2.2). A model of overlapping but opposing receptor subtypes (Szabadi, 1977; Rovati and Nicosia, 1994) was explored as a possible explanation for these observations with capsazepine. Using a logistic equation described by Rovati and Nicosia (1994) and actual maximum responses, EC_{50}, and IC_{50} estimates derived from perfused hindlimb studies with capsaicin, theoretical VO_2 concentration-response curves for putative VN_1 and VN_2 receptors were developed. The sum of these two curves produced a biphasic curve (Chapter 2, Fig. 2.9) that was

very similar to that induced by the infusion of increasing concentrations of capsaicin into the perfused rat hindlimb. Furthermore, the model of Szabadi (1977) predicts that in a dual receptor system where an antagonist has greater affinity for one receptor type, the effect of the opposing receptor will be potentiated, as seen in the studies presented in Chapter 2 using submicromolar concentrations of capsazepine (Fig. 2.2). Similar observations have been made in earlier studies on preparations expressing both α- and β-adrenoreceptors, where β-adrenoreceptor blockade using low antagonist concentrations potentiated the effects of adrenergic agonists (Burks and Cooper, 1967). However, higher antagonist concentrations capable of inhibiting both receptor types were effective at blocking all catecholamine-induced responses in these studies (Burks and Cooper, 1967).

Cooperativity of capsaicin binding to a single vanilloid receptor was considered a possible mechanism to explain the biphasic VO_2 response in perfused hindlimb studies since similar biphasic curves have been observed in radioligand binding studies (Szallasi *et al.*, 1993b, Acs and Blumberg, 1994b). The present studies with the synthetic vanilloid analogue PPAHV, a compound found to bind to vanilloid receptors in a non-cooperative manner (Szallasi *et al.*, 1996), produced very similar results to those seen with capsaicin in this preparation. These findings gave a preliminary indication that cooperative receptor binding is not the cause of the biphasic VO_2 response to capsaicin in the perfused hindlimb. However, in the absence of radioligand binding studies on hindlimb vanilloid receptors, this hypothesis is yet to be proven conclusively. Furthermore, there may be different conformers of the same receptor that display different affinities for capsaicin and related compounds. Indeed, this explanation has been proposed, in favour of a receptor subtype hypothesis, to account for the binding of SP, NKA and septide to NK1 receptors where these compounds do not readily compete with each other in radioligand binding assays (Maggi and Schwartz, 1997). Nonetheless, there is mounting evidence in favour of the existence of vanilloid receptor subtypes in the literature (see section *1.3.2.*) and there is now strong evidence that the perfused rat hindlimb represents a system where these subtypes coexist.

Although the effects of capsaicin in the rat hindlimb are likely to be receptor and cation channel-mediated, it was found that the post-receptor mechanisms are not entirely like those summarised by Maggi (1993) and described in the functional studies of Lou *et al.* (1992). Indeed, the actions of both low and high concentrations of capsaicin were

found to be TTX-resistant in the perfused rat hindlimb. Thus, the dual post receptor mechanisms described by Lou *et al.* (1992) in guinea-pig lung CSPANs are not a likely explanation for the biphasic effect on VO_2 induced by capsaicin in perfused rat muscle. However, the studies presented in Chapter 2 gave little indication as to whether or not the mechanisms of capsaicin action in rat hindlimb involve the stimulation of CSPANs and subsequent release of sensory neuropeptides.

5.1.2. The unique findings revealed by capsaicin pretreatment

The remarkable yet unexpected effects of capsaicin pretreatment shown in Chapter 3 (Fig. 3.5) have further strengthened the idea that the effects of low (VN_1-mediated) and high (VN_2-mediated) concentrations of infused capsaicin occur by separate mechanisms. Of particular interest was the effects induced by high concentrations of capsaicin which showed marked potentiation 7 and 14 days after capsaicin pretreatment, while the effects of low concentrations of capsaicin remained unaltered. Indeed there are very few, if any, reports of capsaicin-mediated effects being augmented after capsaicin pretreatment.

The implications of the studies presented in Chapter 3 are that capsaicin, when infused into the perfused rat hindlimb, stimulates higher affinity vanilloid receptors (VN_1) which release thermogenic (VO_2 stimulating) peptides. These receptors appear to be neuronal (on CSPANs) given that systemic pretreatment with a high dose of capsaicin, known to ablate CSPANs (reviewed by Holzer, 1991; Szolcsányi, 1993), diminishes the acute VO_2 stimulation response to infused capsaicin (Chapter 3, Fig. 3.5). Whether this diminution of the low-dose response was brought about by VN_1 receptor loss or sensory peptide depletion, or both, is unclear from these studies.

An interesting observation made from past perfused hindlimb studies (Cameron-Smith *et al.*, 1990; Colquhoun *et al.*, 1995) is that the perfusion pressure response to infused capsaicin does not show a distinct dual receptor effect (eg. by a point of inflection in the perfusion pressure concentration-response curves). It is plausible that the inflection may be masked by an overlap in the VN_1 and VN_2 responses that produce the same positive effect on vascular tension. However, in the present studies the pretreatment of rats with capsaicin resulted in a marked, selective potentiation of the hindlimb perfusion pressure and VO_2 responses to high capsaicin concentrations (putative VN_2 receptor). Conversely, the vasoconstrictor and VO_2 effects seen at lower

infused concentrations (putative VN_1 receptor) was either inhibited (1 day after pretreatment) or unaltered (7 and 14 days after pretreatment). These findings have provided an initial indication that it may be possible to separate the vasoconstrictor effects induced by nanomolar and micromolar concentrations of capsaicin. Nonetheless, the contribution of each putative receptor subtype to the maximum capsaicin-induced increase in vascular resistance (perfusion pressure) is still unclear as the data presented in this thesis is not entirely consistent with the observations of Colquhoun *et al.* (1995).

The suggestion by Colquhoun *et al.* (1995) that the majority of the capsaicin-induced vasoconstriction was due to the stimulation of the putative VN_1 receptor (see table 2.5) was based on the interesting observation that there was little vasoconstriction but strong inhibition of VO_2 (VN_2 effect) in the absence of external calcium. However, it is possible that the remaining vasoconstriction observed in the absence of external calcium may be sufficient to induce a functional vascular shunt resulting in decreased nutrient delivery and, therefore, reduced hindlimb VO_2. Others have also shown that capsaicin is able to inhibit co-enzymes in the electron transport chain, which may also explain some of the capsaicin-induced inhibition of VO_2 in the absence of strong vasoconstriction (Shimomura *et al.*, 1989; Satoh *et al.*, 1996). In addition, the present study has shown that the competitive antagonist capsazepine, at low concentrations, was able to selectively block the capsaicin-induced stimulation of VO_2 (VN_1 effect) but did not alter the perfusion pressure response (Chapter 2, Fig 2.2). Moreover, submicromolar concentrations of the functional vanilloid antagonist ruthenium red selectively blocked both the inhibition of VO_2 and perfusion pressure responses induced by micromolar concentrations of capsaicin (VN_2 effect) (Chapter 2, Fig. 2.5). In view of these findings, and those from the capsaicin pretreatment studies described above, it appears more likely that the putative VN_2 receptor is responsible for the majority of the vasoconstrictor action of vanilloids in the hindlimb preparation. Alternatively, the above discrepancies may indicate the presence of complex vanilloid receptor expression in muscle, although there is little evidence to support this at present.

5.1.3. The role of the sensory neuropeptides

5.1.3.1. Tachykinins

The stimulation of VO_2 by capsaicin was selectively blocked by non-peptide tachykinin antagonists of NK1 and NK2 receptors (Chapter 3, Figs 3.1 and 3.2), and infused SP, NKA and NKB stimulate VO_2 and mild vasoconstriction with a rank potency order of NKA>NKB>SP (Chapter 3, Fig 3.4). Hence, capsaicin may stimulate VO_2 by releasing endogenous tachykinins which interact primarily with NK2 receptors. The vasoconstriction induced by SP, NKA and NKB in the present study may have resulted from direct stimulation of smooth muscle cell NK2 receptors after diffusion of the peptides across the endothelium. Indeed, others have shown that SP, NKA and NKB are able to induce strong vasoconstriction, in the absence of endothelium, by a mechanism involving the stimulation of smooth muscle NK2 receptors (D'Orléans-Juste *et al.*, 1985; Shirahase *et al.*, 1995). However, in the noradrenalin (NOR)-stimulated perfused hindlimb, SP induced strong but transient vasodilatation (Chapter 4, Figs. 4.4 and 4.5) at concentrations that were ineffective when infused into the unstimulated hindlimb preparation (Chapter 3, Fig. 3.4). This observation may reflect the stimulation of endothelial NK1 receptors by SP to induce vasodilatation that could not be observed in the unstimulated preparation, where an NK2 receptor-mediated vasoconstrictor effect of SP predominates. Indeed, SP has been shown to dilate rat cremaster muscle vasculature by a mechanism that is believed to involve the stimulation of NK1 receptors (Brock and Joshua, 1991). In addition, the vasodilatation induced by stimulation of the rabbit tenuissimus muscle nerve is blocked by the SP antagonist spantide (Persson *et al.*, 1991) and the NK1 receptor antagonist CP 96,345 (Gustafsson *et al.*, 1994). Alternatively, the increase in the potency of SP could be the result of the vascular changes induced by NOR, which is thought to redistribute flow to nutritive vascular beds in muscle (Clark *et al.*, 1995; 1997). The redistribution of flow may account for the observations of Newby *et al.* (1997a), who showed a marked increase in the potency of SP to induce human forearm vasodilatation when co-infused with NOR. However, NKA, which also induces a transient dilatation, shows similar potency in both NOR-stimulated and unstimulated hindlimb preparations (Chapter 4, Figs 4.6 and 4.7), which could involve the stimulation of either NK1 or NK2 receptors given that this peptide retains good affinity for both types (Regoli *et al.*, 1987).

A role for NK1 receptors in the responses to the tachykinins and capsaicin in the perfused hindlimb cannot be excluded since the NK1 receptor antagonist CP-99,994 was able to partly block the effects mediated by capsaicin, although the concentrations used may have been sufficient to block NK2 receptors also. In addition, preliminary autoradiographic studies indicated the presence of specific [^{125}I]-BHSP binding sites in sections of soleus muscle that are likely to correspond to the presence of NK1 receptor in this tissue (see Appendix 1). However, the exact location of these binding sites (eg. vascular endothelium, vascular smooth muscle) was not determined in these studies due largely to the poorly defined skeletal muscle tissue morphology caused by the autoradiographic process. A more refined technique that preserves tissue morphology may involve the use of specific labelled antibodies to NK1 receptors (Vigna *et al.*, 1994).

5.1.3.2. Calcitonin gene-related peptide

Contrary to the actions of the tachykinins, CGRP had no detectable effect on basal VO_2 or pressure when infused into the unstimulated perfused hindlimb (Chapter 3, Fig. 3.4). This apparent lack of effect seen with the infusion of CGRP, a known potent vasodilator, is unlikely to be due to the absence of CGRP receptors in this tissue, but instead probably relates to the lack of vascular tone in this preparation. Indeed, CGRP receptor expression has been demonstrated in cultured L6 rat skeletal muscle cells (Kreutter *et al.*, 1989; Poyner *et al.*, 1992) and whole rat skeletal muscle (Popper and Micevych, 1989; Pittner *et al.*, 1996). In addition, capsaicin elicits vasodilatation in a rat skeletal muscle preparation (cremaster) by stimulating the endogenous release of CGRP (White *et al.*, 1993; Kim *et al.*, 1995) and CGRP itself is able to decrease vascular tone in skeletal muscle (Didion and Mayhan, 1997; Yamada *et al.*, 1997a; 1997b). Hence, it was not unexpected that nanomolar concentrations CGRP elicited a potent vasodilator effect in the perfused hindlimb under NOR-induced vascular tension (Chapter 4, Figs 4.2 and 4.3), a finding that is consistent with its well-documented vasorelaxing properties. This dilatation does not appear to involve the formation of NO as the inhibition of NOS using L-NAME did not alter the vascular response to CGRP, but did inhibit the sustained NO-dependent vasodilatation caused by CCh (Chapter 4, Figs. 4.8 and 4.9). Conversely, the quantitatively and qualitatively similar vascular effects of the tachykinins SP and NKA in the NOR-stimulated perfused hindlimb were

partly sensitive to L-NAME (Chapter 4, Figs. 4.5 and 4.7). This was particularly evident with the NKA-induced effects which showed a reduction in peak dilatation, while both peptides caused strong steady state vasoconstriction when co-infused with the NOS inhibitor.

Further to the dilatation seen with the infusion of CGRP in the NOR-stimulated perfused hindlimb, the CGRP antagonist, $CGRP_{(8-37)}$, enhanced capsaicin-induced vasoconstriction and inhibition of VO_2 in the unstimulated hindlimb (Chapter 3, Fig. 3.3), suggesting that the vasoconstrictor action of capsaicin is opposed by a vasodilator action of endogenous CGRP. Interestingly, similar observations were made in capsaicin-pretreated rats where the vasoconstrictor and inhibitory VO_2 responses to infused capsaicin were enhanced in the perfused hindlimb, indicating that these observations may be due to a reduction in the release of CGRP from sensory neurons.

Thus, in the perfused rat hindlimb, the overall degree of capsaicin-induced vasoconstriction may be the sum of the indirect actions of vasoactive peptides (eg. SP, NKA, CGRP) released from sensory neurons, plus the vasoconstrictor action of capsaicin on vascular smooth muscle via vanilloid receptor stimulation. In cats, "cold storage denervation" potentiates capsaicin-induced vasoconstriction of large cerebral arteries which correlates with degeneration of SP and CGRP-containing perivascular nerves (Saito *et al.*, 1988). These authors suggested that although capsaicin releases vasodilator peptides (presumably SP, CGRP, etc.) from perivascular nerves of cat cerebral arteries, a direct vasoconstrictor effect of capsaicin predominates. This hypothesis is supported by the work of Edvinsson *et al.* (1990) who showed that the vasodilatation induced by capsaicin in cat cerebral arteries was attenuated by repeated capsaicin application or by trigeminal ganglionectomy, whereas the vasoconstrictor effect was unaltered. Similarly, Duckles (1986) has shown that capsaicin applied to the isolated carotid artery and thoracic aorta of the guinea-pig causes vasoconstriction, rather than dilatation, after systemic *in vivo* capsaicin pretreatment. The augmented vasoconstrictor action of capsaicin observed in the perfused hindlimb studies presented in Chapter 3 (Fig 3.5) may also be explained by the absence of sufficient sensory vasodilator peptides after capsaicin pretreatment. However, the studies of Saito *et al.*, Edvinsson *et al.* and Duckles suggest that the vasoconstrictor action of capsaicin occurs by a non-specific effect on the plasma membrane of vascular smooth muscle cells. Conversely, the vascular and metabolic effects of capsaicin in the perfused rat hindlimb

are likely occur via the stimulation of specific vanilloid receptors since both can be blocked by the competitive vanilloid receptor antagonist capsazepine (Chapter 2, Fig 2.2).

5.2. Capsaicin Mechanisms of Action in Perfused Rat Skeletal Muscle

The previous (Cameron-Smith *et al.*, 1990; Colquhoun *et al.*, 1995) and current findings on the vascular and metabolic actions of capsaicin in perfused muscle represent a novel insight into the effects of vanilloids in peripheral tissues. Exactly how capsaicin and the sensory neuropeptides produce their vascular and VO_2 effects in perfused rat muscle is now becoming clearer and is represented in the models shown in figures 5.1 and 5.2. One of the major findings to emerge from these studies is that the actions of capsaicin in this tissue are complex and appear to involve both direct and indirect receptor-mediated effects in the hindlimb preparation. The direct effect of capsaicin appears to involve putative smooth muscle VN_2 receptors which induce strong vasoconstriction and inhibition of VO_2 (Fig. 5.2), while the indirect effect occurs by the more classical vanilloid mechanism involving the stimulation of neuronal (CSPAN) VN_1 receptors and release of sensory neuropeptides (Fig. 5.1). Thus the biphasic effect of capsaicin on hindlimb VO_2 occurs by the stimulation of separate mechanisms. However, unlike the effects of capsaicin in other tissues (e.g. lung) neither mechanism is sensitive to TTX and only one is likely to involve the release of sensory neuropeptides from CSPANs.

Although the tachykinins SP and NKA play at least a partial role in capsaicin-induced stimulation of VO_2 in the perfused hindlimb preparation, the mechanisms underlying this response are not yet fully understood. The concept of site specific vasoconstriction, leading to increased 'nutritive' flow, was summarised in Chapter 1 (section *1.2.2.2)* and has been proposed to explain the large increases in hindlimb VO_2 seen with the infusion of other potent vasoconstrictors, such as NOR, AII and vasopressin (reviewed in Clark *et al.*, 1995; 1997). Based on this flow redistribution model, it appears plausible that submicromolar concentrations of capsaicin may stimulate VO_2 (VN_1 response) by selectively constricting (via a receptor-mediated effect on smooth muscle) or dilating (by release of neuropeptides) blood vessels leading to increased perfusate flow to 'nutritive' vessels (Fig. 5.1). However, a direct effect of

capsaicin and the sensory neuropeptides to stimulate muscle VO_2 cannot be ruled out since, in the present study, NK1 and NK2 receptor antagonists decreased capsaicin-induced stimulation of VO_2, but did not cause appreciable changes in perfusion pressure (Chapter 3, Figs 3.1 and 3.2).

There is convincing evidence that strong vasoconstrictors that inhibit VO_2 in the perfused hindlimb (eg. 5-HT) do so by shunting perfusate away from nutritive vessels to non-nutritive vessels supplying hindlimb connective tissue (septa and tendons) (Newman *et al.*, 1997). Therefore, increased non-nutritive flow may explain the inhibition of VO_2 that accompanies the strong vasoconstriction induced by high concentrations of capsaicin. This hypothesis is strengthened by the observation that the augmentation of capsaicin-induced vasoconstriction 1, 7 and 14 days after capsaicin pretreatment (Chapter 3, Fig. 3.5) produced a concomitant potentiation of VO_2 inhibition.

It seems unlikely that endogenously released tachykinins would have a major role in producing the steady state changes in vascular tone induced by capsaicin given the transient nature of the vasodilatation seen with the infusion of these peptides in the present studies (see Chapter 4). However, the sustained vasodilator effect of CGRP in the NOR-stimulated perfused hindlimb (Chapter 4, Figs. 4.2 and 4.3) is in good agreement with the findings in Chapter 3 where $CGRP_{(8\text{-}37)}$ augmented the vasoconstrictor effect of capsaicin, further supporting a vasodilator role for endogenously released CGRP (Chapter 3, Fig. 3.3). Moreover, the present results in the NOR-stimulated perfused hindlimb further support the notion that capsaicin is a potent vasoconstrictor in this tissue (Chapter 4, Figs. 4.10 and 4.11). Thus, while capsaicin probably stimulates the release of potent vasodilator peptides, such as CGRP, the vasoconstrictor action of this pungent spice principle is the predominant effect in the perfused rat hindlimb.

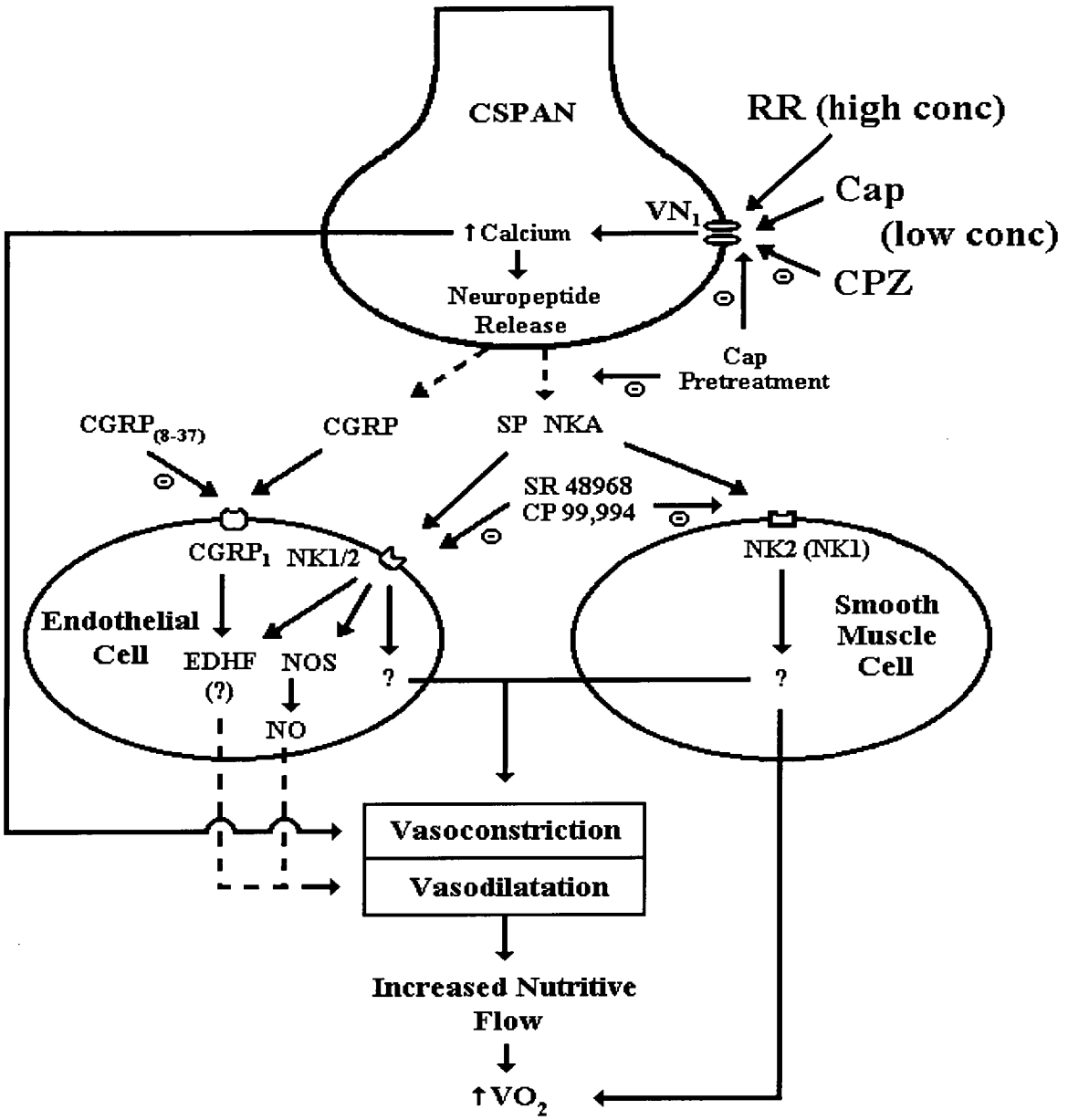


Fig. 5.1. Proposed mechanisms of action for the vascular and VO_2-stimulating effects of capsaicin in the perfused rat hindlimb via stimulation of putative VN_1 receptor/channels. This subtype shows high sensitivity to capsazepine (CPZ) but low sensitivity to ruthenium red (RR). The VN_1-mediated mechanism is likely to involve CSPANs given that capsaicin pretreatment ablates the stimulatory response on VO_2. VN_1 stimulation may have a direct vasoconstrictor effect and also stimulates the release of neuropeptide transmitters which induce vasodilatation and/or vasoconstriction. These vascular effects are likely to increase nutritive muscle flow and, therefore, increase VO_2. A direct VO_2-stimulating effect of the tachykinins cannot be ruled out since non-peptide tachykinin receptor antagonists (CP-99,994 or SR 48968) partly blocked capsaicin-induced VO_2, but not PP. Broken arrows indicate release or neuromodulators or endothelium-derived factors, solid arrows show consequence.

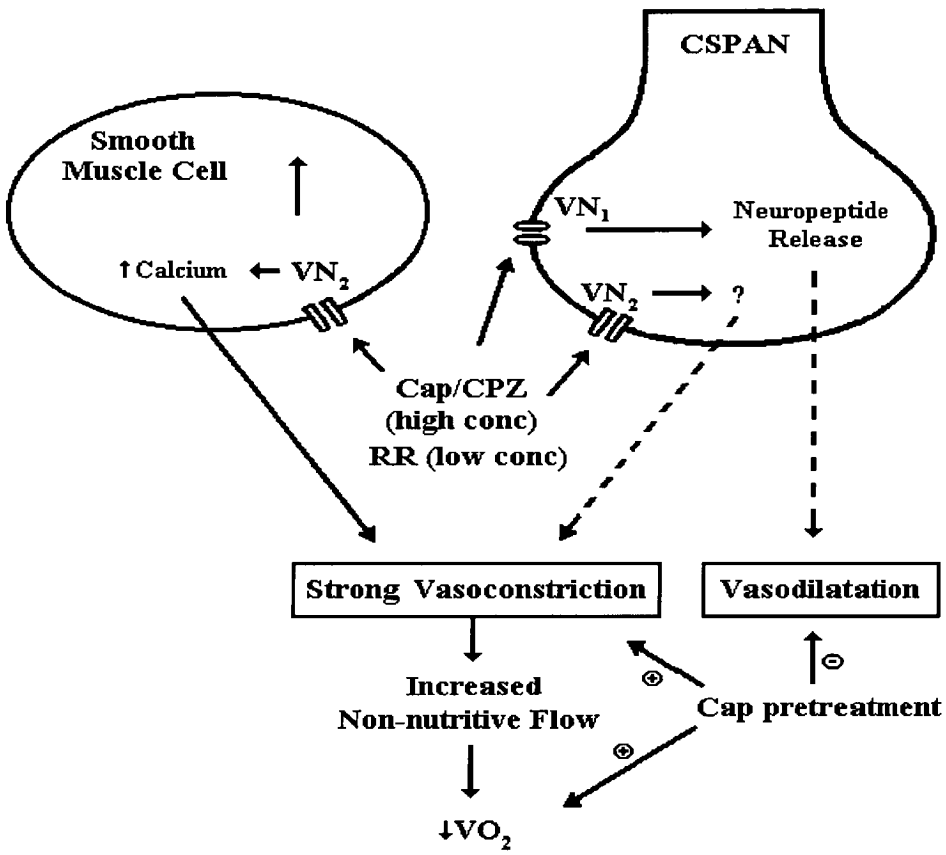


Fig. 5.2. Proposed mechanisms of capsaicin-induced vasoconstriction and inhibition of VO_2 via stimulation of the putative muscle VN_2 receptor. VN_2 receptors are possibly non-neuronal (smooth muscle) given that high concentrations of capsaicin induce strong vasoconstriction which is enhanced, rather than inhibited, by capsaicin pretreatment. Alternatively, VN_2 may represent a previously unidentified type of neuronal vanilloid receptor/channel that is not subject to tachyphylaxis with capsaicin pretreatment, has low sensitivity to capsazepine (CPZ), and high sensitivity to ruthenium red (RR). It is proposed that the strong vasoconstriction induced by high concentrations of infused capsaicin, acting via VN_2 receptors, increases non-nutritive flow in the perfused hindlimb preparation, thus decreasing VO_2. Potentiation of the observed VN_2-mediated vasoconstriction and VO_2 inhibition after capsaicin pretreatment is likely to be due to the depletion of vasodilator neuropeptides, or may result in upregulation of VN_2 receptors or receptor-coupling mechanisms. Broken arrows indicate neuromodulator release, solid arrows show consequence.

5.3. Vanilloid Receptor Nomenclature

The data presented in this thesis, and the work of others in the perfused rat hindlimb (Colquhoun *et al.*, 1995), has added further to the mounting evidence in favour of vanilloid receptor subtypes from radioligand binding studies, Ca^{2+} uptake studies, and whole cell patch-clamp studies on rat DRG and trigeminal ganglion neurons. Although quite compelling, such evidence is indirect and it is difficult to estimate the true number of vanilloid receptor subtypes at present. However, the recent breakthrough in vanilloid receptor cloning (Caterina *et al.*, 1997) may lead to a rapid proliferation of knowledge in this area and, potentially, the cloning of receptor subtypes distinct from those already cloned from rat DRG neurons.

In view of the results in Chapter 3 showing reduced responses to low concentrations of infused capsaicin 1 day after capsaicin pretreatment, it follows that the putative VN_1 receptor may be expressed on CSPANs in this tissue. As these neurons have their cell bodies in the DRG, it is plausible to suggest that this receptor is similar to the VR_1 vanilloid receptor cloned by Caterina *et al.* (1997) from DRG neurons. However, a large body of evidence obtained from the radioligand binding studies of Szallasi, Blumberg and their co-workers have shown that peripheral vanilloid receptors display non-cooperative binding characteristics while those that are central in origin, such as in the DRG, bind cooperatively (review by Szallasi, 1994). The studies using 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) presented in Chapter 2 (Fig. 2.6) indicate that capsaicin binds to hindlimb vanilloid receptors in a non-cooperative manner and therefore displays characteristics that are likely to be different from the cloned CNS VR_1 receptor. Without further characterisation of muscle vanilloid receptors by radioligand binding and/or molecular cloning studies it is difficult to assign these receptors to putative subtypes proposed by others.

Whereas vanilloid receptors described by others are neuronal in origin and are subject to tachyphylaxis, the putative VN_2 receptor subtype may be a non-neuronal, smooth muscle vanilloid binding site. This is based on the evidence presented in Chapter 3 where VN_2-mediated effects were not attenuated by capsaicin pretreatment and appear to be largely responsible for the strong vasoconstrictor effect of capsaicin in the hindlimb preparation. Hence, this receptor may be unlike any other vanilloid receptor described previously, but again this hypothesis requires confirmation with

receptor binding and/or molecular cloning studies. Alternatively, the muscle VN_2 receptor may represent a subtype of neuronal vanilloid receptor that displays reduced susceptibility to desensitisation. Indeed, recent patch-clamp studies in rat trigeminal ganglia with the synthetic vanilloid analogue PPAHV have indicated the presence of vanilloid receptor subtypes with different activation and desensitisation kinetics (Liu *et al.*, 1998). With the membrane potential held at -60 mV, rapidly-activating inward currents induced by PPAHV were eliminated by repeated applications of 3 μM of the synthetic analogue, whereas distinct slowly-activating currents were only partly reduced. Moreover, some of the currents induced by PPAHV were insensitive to treatment with capsazepine providing further evidence for vanilloid receptor subtypes on these neurons (Liu *et al.*, 1998). However, it was not clear from these studies which currents (i.e. fast- or slow-activating) were sensitive and which were insensitive to the vanilloid antagonist.

5.4. Vanilloid effects *in vivo*

The systemic administration of capsaicin is well known for its excitatory and long-lasting analgesic effect at high doses (greater than 10 mg/kg), the latter resulting from functional and neurotoxic impairment of CSPANs (reviewed by Holzer, 1991; Szolcsányi, 1993). However, other *in vivo* actions of capsaicin have been described in rat tissues where sensory neurons play an important regulatory role and where capsaicin induces cell non-selective effects (reviewed by Campbell *et al.*, 1993). Briefly, these effects include a reduction in core body temperature (Hayes *et al.*, 1984), effects on the airways including bronchial and tracheal constriction (reviewed by Lundberg, 1993), contraction of the urinary bladder (reviewed by Maggi, 1992), as well as inhibitory effects on cardiac muscle (Franco-Cereceda and Lundberg, 1988). When administered systemically, capsaicin has other profound effects on the cardiovascular system in the rat. Cardiovascular responses to the acute administration of capsaicin differ depending on the strain of rat studied, however a vagally mediated reduction blood pressure has been consistently observed (Donnerer and Lembeck, 1982; Chahl and Lynch, 1987, Campbell *et al.*, 1993). Others have shown that this reflex fall in blood pressure induced by capsaicin is inhibited by the intrathecal administration of an SP antagonist

suggesting that the release of SP from the CNS is involved in the response (Donnerer and Lembeck, 1983).

An important consideration to draw from studies on the vascular and metabolic effects of capsaicin in the perfused rat hindlimb (Cameron-Smith *et al.*, 1990; Eldershaw *et al.*, 1992; 1994; Colquhoun *et al.*, 1995; Chapters 2, 3 and 4) is whether or not these actions are likely to occur *in vivo,* and whether or not they have any physiological significance. The studies presented in Chapter 4 in the NOR-stimulated perfused hindlimb indicate that capsaicin is still a potent vasoconstrictor in skeletal muscle at near physiological vascular tone (Figs. 4.10 and 4.11). However, the actions of NOR itself on hindlimb nutritive/non-nutritive flow distribution must be considered (reviewed by Clark *et al.*, 1995; 1997) as this may not be the same as the flow distribution *in vivo*. Furthermore, NOR has a potent action on hindlimb muscle VO_2 making it difficult to draw conclusions regarding the likely action of capsaicin on muscle metabolism *in vivo*. Nonetheless, others have shown that the *in vivo* administration of capsaicin has a vasoconstrictor effect in the autoperfused hindlimbs of the dog where basal perfusion pressure is held at a physiological level (Webb-Peploe *et al.*, 1972; Pelletier and Shepherd, 1975). It has also been shown that the vasoconstrictor effect in the dog hindlimb seen with the injection of capsaicin into the iliac arteries was not affected by sciatic or femoral nerve sectioning, while the concomitant reflex vasoconstriction seen in the aorta and splenic vessels was attenuated (Webb-Peploe *et al.*, 1972). Thus the hindlimb vasoconstriction was due to local stimulation by capsaicin rather than an axon reflex, as shown in the perfused rat hindlimb (Cameron-Smith *et al.*, 1990). Interestingly, it has been suggested that the reflex cardiovascular responses seen with the activation of capsaicin-sensitive skeletal muscle receptors may be the same receptors that are activated during muscular contraction to cause a redistribution of blood flow to working skeletal muscle (Webb-Peploe *et al.*, 1972; Crayton *et al.*, 1981). Thus an *in vivo* function may potentially be ascribed to skeletal muscle vanilloid receptors involving reflex cardiovascular responses to exercise in addition to local metabolic and vascular effects. However, it is unclear which of the putative muscle vanilloid receptors (i.e. VN_1, VN_2 or both) are likely to be involved in the reflex cardiovascular responses. Capsaicin has been shown to activate group IV (C-type) afferent fibres in hindlimb muscles of the dog (Kaufman *et al.*, 1982) which may indicate the involvement of neuronal VN_1 receptors, although such suggestions are

purely speculative at present. It is interesting to note that these authors showed a resistance of muscle C-type afferents to the acute tachyphylactic response normally induced by repeated capsaicin application. A similar resistance to acute vanilloid desensitisation has been observed in the perfused rat hindlimb (Eldershaw *et al.*, 1994).

5.5. Therapeutic Potential of Vanilloids

There are few reports describing the effects of capsaicin and other vanilloids on oxygen consumption in individual tissues. Understanding how vanilloids alter skeletal muscle metabolism is essential for the use of these compounds as therapeutic agents for the stimulation of whole body oxygen consumption and thermogenesis. The likelihood that skeletal muscle metabolism may be controlled by stimulatory (VN_1) and inhibitory (VN_2) receptors may provide a means by which whole body energy expenditure can be increased by synthetic vanilloids that selectively stimulate VN_1 receptors, or selectively inhibit VN_2 receptors in the presence of capsaicin. Preliminary *in vitro* and *in vivo* structure-activity investigations using a synthetic vanilloid agents have shown favourable thermogenic activity, however all of the synthetic compounds tested were unable to selectively stimulate putative hindlimb VN_1 or VN_2 receptors (T.P.D. Eldershaw, PhD Thesis, University of Tasmania, 1996). Nonetheless, the potential of these compounds in the treatment of metabolic disorders is encouraging and is compounded by recent preliminary experiments showing that hindlimb insulin-mediated glucose uptake (IMGU) in the rat can be acutely influenced by capsaicin (T.P.D. Eldershaw, E.Q. Colquhoun and C.D. Griffiths, unpublished observations). Indeed other vasoconstrictors that induce similar effects to capsaicin on hindlimb VO_2 also affect muscle IMGU in a similar manner (Rattigan *et al.*, 1993; 1995; 1996). Thus vanilloids may also have implications for the treatment of other human disorders where defective muscle vascular control may have pathogenic implications.

The *in vivo* actions of capsaicin described above (section *5.4*) raise concerns regarding its use as a therapeutic agent and encourage the need for the development of synthetic vanilloid agents that are devoid of nociceptive and other undesirable side-effects. Interest in vanilloids as non-steroidal anti-inflammatory agents has focused more recently on the *Euphorbium* spice principle RTX, a compound that is less likely to induce unwanted cardiovascular and respiratory reflexes (Appendino and Szallasi,

1997). RTX also has the added advantages of high potency and a greater efficacy for inducing desensitisation than for inducing pain (reviewed by Blumberg *et al.*, 1993; Appendino and Szallasi, 1997). However, the promotion of this ultrapotent capsaicin analogue as a therapeutic agent has also been hampered by the undesired side-effects caused by its administration *in vivo*. Most notably, RTX causes a profound reduction in body temperature at doses required to induce analgesia (Szallasi and Blumberg, 1989). More recently, the development of synthetic phorbol-based vanilloids has yielded encouraging compounds (eg. PPAHV) which are only mildly pungent and fail to induce a measurable hypothermic response at doses that protect against neurogenic inflammation (Appendino *et al.*, 1996).

Earlier structure-activity studies with synthetic vanilloid analogues possessing long chain C-regions identified promising compounds such as olvanil which showed reduced or abolished activity in pungency assays, but retained antinociceptive and 'desensitising' properties (Brand *et al.*, 1987). Modification of this compound by aromatic ring substitution produced nuvanil, an agent that showed improved solubility but, like olvanil, only produced analgesic effects at oral-administered doses where profound hypothermic effects were observed (Campbell *et al.*, 1993). Subsequent structure-activity studies by Walpole and coworkers have delineated the structural features of the capsaicin molecule that are necessary for agonist and antinociceptive activity (Walpole *et al.*, 1993a-c). Combinations of the optimal structural features from the three regions of the capsaicin molecule have recently yielded a series of highly potent agonists with improved *in vivo* pharmacokinetic profiles (Wrigglesworth *et al.*, 1996). Thus, these molecules represent potential candidates for the development of oral analgesic agents and may overcome the problems of natural analogues such as capsaicin and dihydrocapsaicin, and synthetic analogues such as olvanil, which are rapidly metabolised before entering the systemic circulation (Donnerer *et al.*, 1990; Sietsema *et al.*, 1988). It would be interesting to determine the effects of these compounds on muscle and/or whole body thermogenesis to examine their potential for development as orally-administered stimulators of metabolism.

5.6. Future Directions for Vanilloid Studies

Within the vanilloids field of research there is undoubtedly an urgent need to gain further understanding of vanilloid receptor subtype diversity. The cloning of a DRG vanilloid receptor (Caterina *et al.*, 1997) has promoted further interest and excitement in this field that may rapidly lead to the cloning of an entire new receptor family. If vanilloid receptor subtypes do exist then it follows that the genes encoding these proteins may occupy different loci. It would also be likely that different genes encoding the various vanilloid receptor subtypes would contain highly conserved regions that may be potential targets for the development of specific DNA probes, or primers to enable amplification of the genes by polymerase chain reaction (PCR) techniques. Indeed, the study of vanilloid pharmacology may follow a similar path to that of the tachykinins where to the cloning and recognition of receptor subtypes for these neuromodulators has contributed significantly to the understanding of tachykinin pharmacology (Yokota *et al.*, 1989; Hershey *et al.*, 1991; Ingi *et al.*, 1991). Furthermore, it has permitted a greater understanding of the binding characteristics of selective agonists and antagonists for NK1, NK2 and NK3 receptors, as well as enabling the development of specific receptor antibodies for improved pharmacological studies (Vigna *et al.*, 1994). Such knowledge would be highly valuable for the development of selective ligands or antagonists for the putative VN_1 and VN_2 hindlimb receptors.

It is a widely accepted that any pharmacological study attempting to conclusively prove the existence of a receptor family or type, that three lines of evidence are important: a) cloning of the receptor(s) and identification of the gene(s) responsible for its production; b) radioligand binding and/or autoradiographic studies for tissue distribution analysis and determination of receptor kinetics; and c) functional studies to characterise the receptor(s), and to ascribe it a physiological function. While all three forms have now been described for DRG vanilloid receptors, only the latter two have been studied with respect to peripheral vanilloid receptors, and only the final criterion has been studied with regards to rat hindlimb vanilloid receptors. For studies in rat skeletal muscle, radioligand binding experiments should confirm the presence of specific [^{3}H]-RTX (vanilloid) binding sites and may further add to the evidence in favour of vanilloid receptor subtypes in this tissue. Competition binding studies using capsaicin analogues and capsazepine should reveal more about the binding kinetics of

these compounds to vanilloid receptors (i.e. cooperative versus non-cooperative binding behaviour). In addition, autoradiographic studies on skeletal muscle will reveal more about the tissue distribution of these novel binding sites, and since capsazepine binds selectively to putative VN1 receptors at low concentrations (Chapter 2, Fig 2.2), this antagonist may enable the determination of VN_1/VN_2 receptor locations.

The encouraging positive results obtained with synthetic vanilloids *in vivo* (T.P.D. Eldershaw, PhD Thesis, University of Tasmania, 1996) warrants further investigation to confirm the potential of capsaicin-like molecules as whole-body metabolic stimulators. If our future knowledge of vanilloid receptors permits the synthesis of selective VN_1 agonists or VN_2 antagonists then *in vivo* studies in the rat will be of great importance for the development of thermogenic drugs for subsequent trials in humans. An important consideration in the structural design of compounds for future *in vivo* studies is their bioavailability after oral administration, given the reported metabolic degradation of capsaicin-like molecules (see section *5.5*).

5.7. Conclusions

The mechanisms underlying the vascular and metabolic effects of vanilloids in perfused rat skeletal muscle have been further defined. The studies undertaken support the notion of putative vanilloid receptor subtypes controlling the biphasic VO_2 effect, and the alterations in vascular tone, induced by capsaicin and structurally related compounds. The effects of capsaicin in this tissue may result from a combination of NO-dependent and –independent vasodilatation, vasoconstriction and VO_2 stimulation induced by endogenous sensory neuropeptides, and the overriding vasoconstrictor effect of capsaicin via vanilloid receptor stimulation. It is likely that the direct and indirect vascular effects of these compounds are able to alter the distribution of nutrient access to muscle, thus altering resting metabolic rate. However an effect of capsaicin and its neuromodulators to directly alter muscle metabolism cannot be ruled out. These findings underline the complex nature of capsaicin's novel actions in perfused rat skeletal muscle.

It is not yet clear whether the favourable thermogenic effects of capsaicin seen in the perfused hindlimb preparation will also result in the stimulation of muscle, and whole body, thermogenesis *in vivo*. Thus, these compounds remain potential therapeutic agents for the treatment of metabolic disorders. Nonetheless, the perfused

rat hindlimb studies have revealed a very interesting discovery in terms of vanilloid receptor nomenclature. That is, the presence of putative vanilloid receptor subtypes in muscle which can, potentially, be exploited to favourably alter skeletal muscle metabolism.

Appendix 1

NK1 receptor autoradiography

The results presented in Chapter 3 suggest that the vasoconstriction and stimulation of VO_2 by capsaicin in the perfused rat hindlimb may be partly mediated by endogenous tachykinins acting on NK2 receptors. However, a role for NK1 receptors cannot be dismissed since the NK1 receptor antagonist CP-99,994 partly blocked the stimulation of VO_2 produced by capsaicin (Chapter 3, Fig. 3.1). Hence, the present studies have attempted to establish the presence of NK1 receptors in skeletal muscle tissue by examining [^{125}I]-Bolton-Hunter substance P ([^{125}I]-BHSP) binding in rat soleus muscle sections. Furthermore, the effects of capsaicin pretreatment on [^{125}I]-BHSP binding, previously shown to increase [^{125}I]-BHSP binding in guinea-pig *vas deferens* (Mussap *et al.*, 1989), was examined in this tissue.

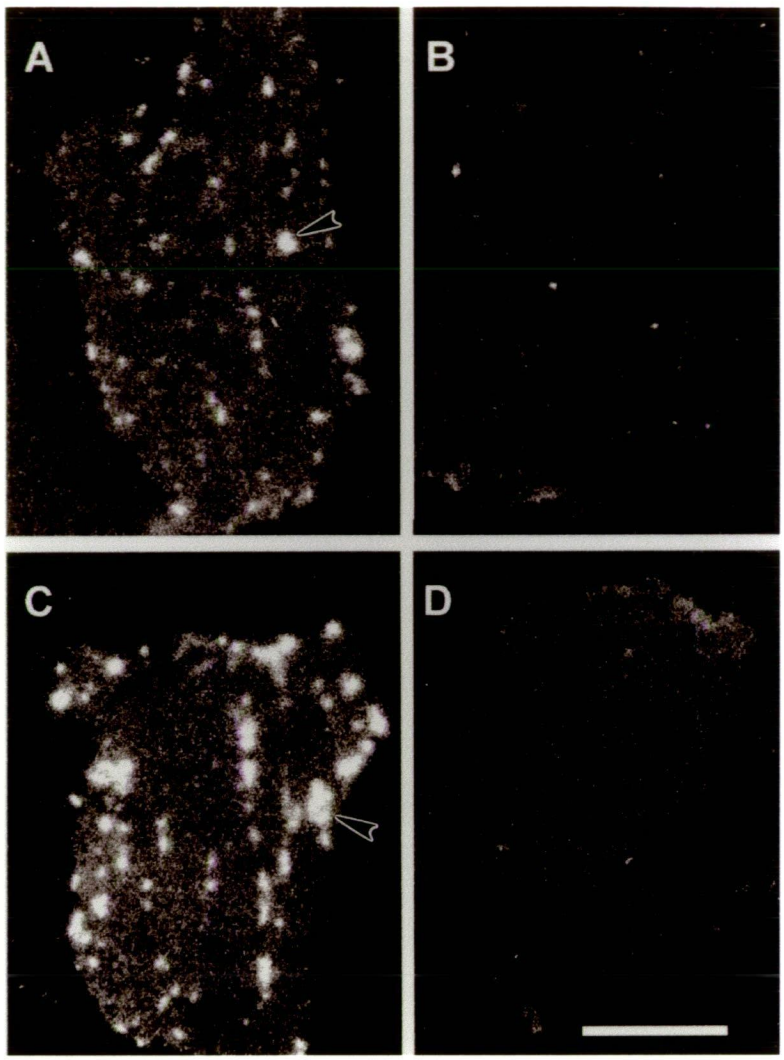
[^{125}I]-BHSP (2,200 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). Chymostatin, leupeptin, bacitracin, bestatin and phosphoramidon were purchased from the Sigma Chemical Company. Tachykinin peptides were obtained from the sources mentioned in section 3.2.2.

Longitudinal sections (15 μM) of tissue were cut from the soleus muscles of vehicle and capsaicin pretreated animals (see Chapter 3, section *3.2.4* for pretreatment protocol) using a cryostat (Leica, Germany) set at -16 to -18°C. Sections were thaw-mounted onto gelatine-coated glass slides in duplicate, and desiccated overnight under vacuum to be used immediately, or stored at -70°C for later use. Autoradiographic studies were performed using the method of Lew *et al.* (1990). Sections were equilibrated to room temperature and preincubated twice for 5 minutes in 170 mM Tris buffer (pH 7.4, 25°C), containing 0.02% BSA, to remove bound endogenous tachykinins. A further 10 minute incubation in the above buffer containing $MnCl_2$ (3 mM), bacitracin (40 $\mu g.ml^{-1}$), chymostatin (4 $\mu g.ml^{-1}$), leupeptin (4 $\mu g.ml^{-1}$), bestatin (10 μM), and phosphoramidon (5 μM) ensured the inhibition of endogenous peptidases. Sections were then incubated for 45 minutes with 90 pM [^{125}I]-BHSP, washed (4 x 2 minutes) in ice-cold 170 mM Tris (pH 7.4, 4°C) with 0.02% BSA and $MnCl_2$ (3 mM), then rinsed in ice-cold distilled water. Non-specific binding was determined by incubating sections with [^{125}I]-BHSP in the presence of 1 μM unlabelled SP.

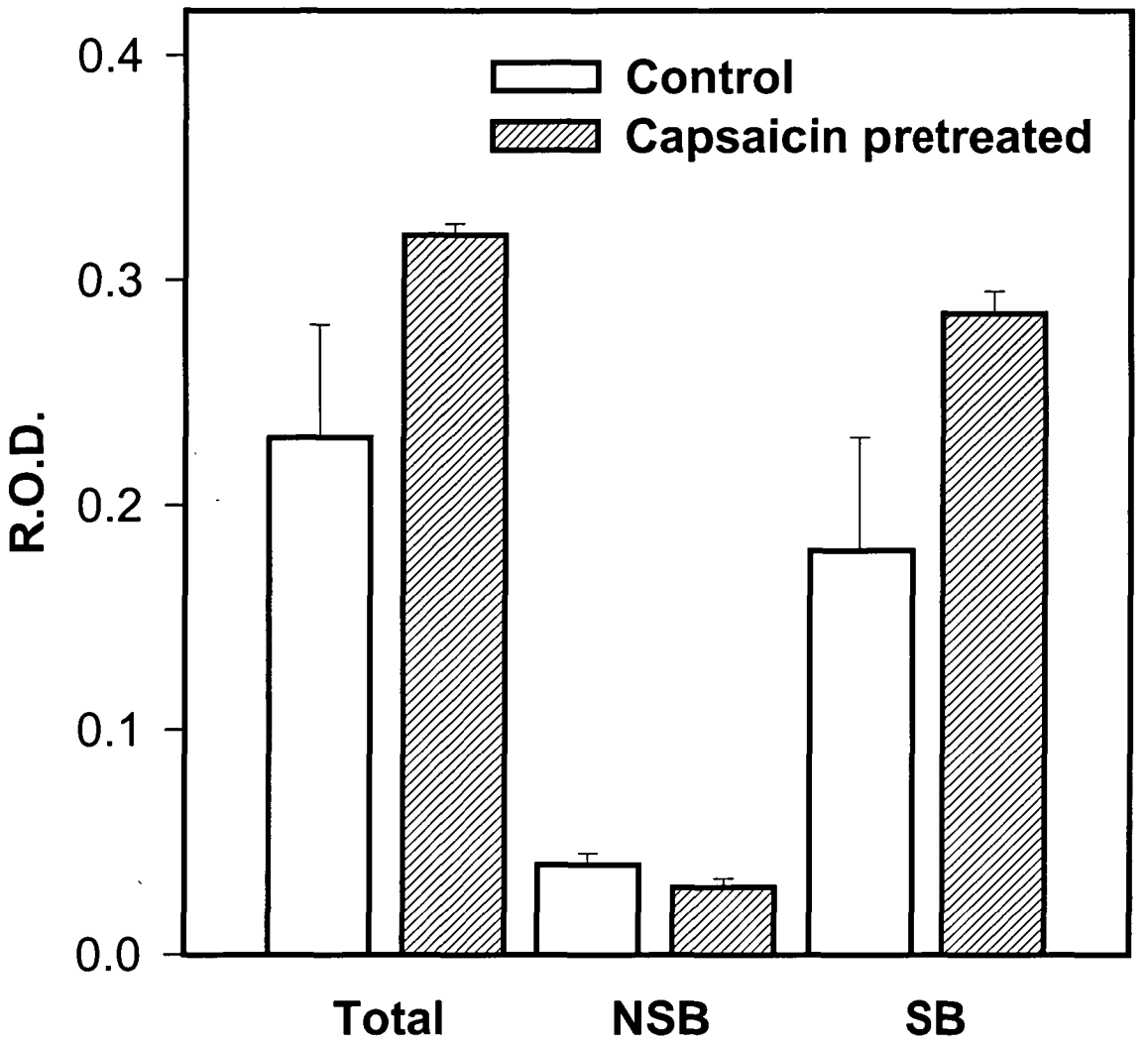
Labelled sections were dried and apposed to ^{3}H Hyperfilm (Amersham, Buckinghamshire, UK) for 3 weeks at 4°C. Hyperfilms were developed under light-safe conditions in Ilford Phenisol x-ray developer (4.5 minutes at 7°C), and fixed in Ilford Hypam x-ray rapid fixer containing Ilford Hypam x-ray hardener (5 minutes at 25°C). The films were then washed, dried and used as negatives to generate corresponding positive black and white photographic prints.

Figure 1 shows the presence of localised, specific binding of [^{125}I]-BHSP to rat soleus muscle sections that may indicate the presence of NK1 receptors in this tissue. Histological examination of the sections suggested that the binding was associated with blood vessels (data not shown). However, the morphology of the tissue was poor and it was not possible to determine whether the binding was located on the endothelium, vascular smooth muscle, or both.

Multiple measurements of relative optical density (R.O.D) were made over areas where binding was localised, in sections from both vehicle and capsaicin pretreated rats, using Analytical Imaging Station (AIS) software (Fig. 2). Mean values (± s.e.) were calculated for each set of sections from individual animals and these show an increase in specific [^{125}I]-BHSP binding in soleus muscle sections from rats pretreated with capsaicin. However, this increase was not statistically significant by Student's t-test ($P<0.05$), an observation that may be due to the low number of experiments performed. Nonetheless, these findings are similar to those of Mussap *et al.* (1989) who suggested that the increase in [^{125}I]-BHSP binding in the outer longitudinal smooth muscle of the guinea-pig *vas deferens* may have been due NK1 receptor upregulation in response to sensory neuron loss.



Appendix 1 Fig.1. Photomicrographs taken directly from ^{3}H-Hyperfilm images of [^{125}I]-Bolton-Hunter substance P ([^{125}]-BHSP) binding to longitudinal sections of soleus muscle obtained from rats pretreated 14 days previously with vehicle or capsaicin. White represents areas of high binding density (arrows). Panels A and B show total and non-specific binding, respectively, in vehicle pretreated controls, whereas panels C and D show total and non-specific binding, respectively, in capsaicin pretreated rats. Note that non-specific binding is uniformly low for both treatment groups, whereas total binding appears to be greater in the capsaicin pretreated rats. Bar = 1 mm.



Appendix 1 Fig. 2. Multiple measurements of relative optical density (R.O.D.) of total, non-specific (NSB) and, therefore, specific (SB) [^{125}I]-Bolton-Hunter substance P ([^{125}I]-BHSP) binding to longitudinal sections of soleus muscle obtained from rats pretreated 14 days previously with vehicle or capsaicin. Measurements were made over areas of localised binding from 2-3 experiments.

Appendix 2

Weight trials of animals pretreated systemically with capsaicin (125 mg. kg^{-1}) or vehicle (10% Tween 80, 10% ethanol in normal saline) over the 3-day injection protocol, and just prior to perfusion (final weight). Experimental methods are given in detail in Chapter 3, section *3.2.4. Capsaicin pretreatment*.

Pretreatment group	Pretreatment type	[1]n	Weight during protocol (g)			[2]Final weight (g)
			Day 1	Day 2	Day 3	
1 Day	Vehicle	5	173.0±6.1	177.2±6.5	171.8±5.6	171.0±4.2
	Capsaicin	5	178.2±5.0	180.0±5.4	166.0±6.8	168.0±4.2
7 Days	Vehicle	5-6	151.8±2.3	154.3±3.4	152.8±3.3	194.0±1.8
	Capsaicin	5-6	157.7±2.2	160.0±3.2	145.2±3.4*†	196.2±2.9
14 Days	Vehicle	5-9	97.8±1.0	103.0±1.6*	101.1±1.6	187.4±5.2
	Capsaicin	5-9	104.1±1.3	104.7±2.1	96.0±2.4**†	184.0±4.5
Total	Vehicle	15-20	132.8±7.7	138.7±7.7	136.1±7.4	184.1±3.4
	Capsaicin	15-20	138.7±7.5	144.1±8.1	133.3±8.1	182.7±3.7

Values are mean ± s.e. mean. *P<0.05 **P<0.01 from day 1 weight. †P<0.05 from day 2 weight (Student's t-test).

[1]Number of animals vary due to a 25% total mortality rate during pretreatment protocol.

[2]Final weight not included in statistical analysis.

References

ACS, G. AND BLUMBERG, P.M. (1994a) Comparison of [^{3}H]-resiniferatoxin binding to spinal cord and dorsal root ganglia of newborn and adult rats. *Life Sci.* **54**: 1875-1882.

ACS, G. AND BLUMBERG, P.M. (1994b) [^{3}H]-Resiniferatoxin binding to pig dorsal horn membranes displays positive cooperativity. *Life Sci.* **55**: 337-346.

ACS, G., PALKOVITS, M. AND BLUMBERG, P.M. (1994a) [^{3}H]-resiniferatoxin binding by the human vanilloid (capsaicin) receptor. *Brain Res. Molec. Brain Res.* **23**: 185-190.

ACS, G., PALKOVITS, M. AND BLUMBERG, P.M. (1994b) Comparison of [^{3}H]-resiniferatoxin binding by the vanilloid (capsaicin) receptor in dorsal root ganglia, spinal cord, dorsal vagal complex, sciatic and vagal nerve and urinary bladder of the rat. *Life Sci.* **55**: 1017-1026.

ACS, G., LEE, J., MARQUEZ, V.E. AND BLUMBERG, P.M. (1996) Distinct structure-activity relations for stimulation of ^{45}Ca uptake and for high affinity binding in cultured rat dorsal root ganglion neurons and dorsal root ganglion membranes. *Brain Res Molec. Brain Res.* **35**: 173-182.

ADVENIER, C. (1995) Tachykinin NK2 receptors further characterised in the lung with non-peptide receptor antagonists. *Can. J. Physiol. Pharmacol.* **73**: 878-884.

AKOPIAN, A.N., SIVILOTTI, L. AND WOOD, J.N. (1996) A tetrodotoxin-resistant voltage-gated sodium channel expressed in sensory neurons. *Nature* **379**: 257-262.

ALLOGHO, S.N., NGUYENLE, X.K., GOBEIL, F., PHENG, L.A. AND REGOLI, D. (1997) Neurokinin receptors (NK1, NK2) in the mouse – a pharmacological study. *Can. J. Physiol. Pharmacol.* **75**: 552-557.

AMANN, R. AND MAGGI, C.A. (1991) Ruthenium red as a capsaicin antagonist. *Life Sci.* **49**: 849-856.

AMANN, R., DONNERER, J. AND LEMBECK, F. (1989) Ruthenium red selectively inhibits capsaicin-induced release of calcitonin gene- related peptide from the isolated perfused guinea pig lung. *Neurosci.Lett.* **101**: 311-315,.

AMANN, R., DONNERER, J., MAGGI, C.A., GIULIANI, S., DELBIANCO, E., WEIHE, E. AND LEMBECK, F. (1990) Capsaicin desensitization *in vivo* is inhibited by ruthenium red. *Eur.J.Pharmacol.* **186**: 169-175.

APPENDINO, G., CRAVOTTO, G., PALMISANO, G., ANNUNZIATA, R AND SZALLASI, A. (1996) Synthesis and evaluation of phorboid 20-homovanillates: discovery of a class of ligands binding to the vanilloid (capsaicin) receptor with different degrees of cooperativity. *J. Med. Chem.* **39**: 3123-3131

APPENDINO, G. AND SZALLASI, A. (1997) Euphorbium: modern research on its active principle, resiniferatoxin, revives an ancient medicine. *Life Sci.* **60**: 681-696.

AUSTRALIAN GOVERMENT. (1990) *Australian code of practice for the care and use of animals for scientific purposes.* Australian Government Publishing Service, Canberra.

BELL, D. AND MCDERMOTT, B. (1996) Calcitonin gene-related peptide in the cardiovascular system: characterisation of receptor populations and their (patho)physiological significance. *Pharmacol. Rev.* **48**: 253-288.

BEVAN, S.J. AND DOCHERTY, R.J. (1993) Cellular mechanisms of the action of capsaicin. In: *Capsaicin in the study of pain.* Wood, J.N. (ed.), pp. 27-44, Academic Press Ltd, London.

BEVAN, S. AND FORBES, C.A. (1988) Membrane effects of capsaicin on dorsal root ganglion neurones in cell culture. *J. Physiol.* **328**: 28P.

BEVAN, S. AND SZOLCSÁNYI, J. (1990) Sensory neuron-specific actions of capsaicin: mechanisms and applications. *Trends Pharmacol. Sci.* **11**: 330-333.

BEVAN, S., HOTHI, S., HUGHES, G., JAMES, I.F., RANG, H.P., SHAH, K., WALPOLE, C.S.J., AND YEATS, J.C. (1991) The development of a competitive antagonist for the sensory neuron excitant capsaicin. *Br. J. Pharmacol.* **102**: 77P

BEVAN, S., HOTHI, S., HUGHES, G., JAMES, I.F., RANG, H.P., SHAH, K., WALPOLE, C.S.J., AND YEATS, J.C. (1992) Capsazepine: a competitive antagonist of the sensory neurone excitant capsaicin. *Br. J. Pharmacol.* **107**: 544-552.

BLUMBERG, P.M., SZALLASI, A. AND ACS, G. (1993) Resiniferatoxin: an ultrapotent capsaicin analogue. In: *Capsaicin in the study of pain.* Wood, J.N. (ed.), pp. 45-62, Academic Press Ltd, London.

BONEN, A., CLARK, M.G. AND HENRIKSEN, E.J. (1994) Experimental approaches in muscle metabolism: hindlimb perfusion and isolated muscle incubations. *Am. J. Physiol.* **266**: E1-E16.

BOWDEN, J., GARLAND, A., BALUK, P., LEFEVRE, P., GRADY, E., VIGNA, S., BUNNETT, N. AND MCDONALD, D. (1994) Direct observation of substance P-induced internalisation of neurokinin 1 (NK1) receptors at sites of inflammation. *Proc. Natl. Acad. Sci. U.S.A* **91**: 8964-8968.

BRAIN, S.D. (1996) Sensory neuropeptides in the skin. In: *Neurogenic Inflammation*, Geppetti, P. and Holzer, P. (eds.) pp. 229-244, CRC Press, Boca Raton, FL.

BRAND, L., BERMAN, E.F., SCHWEN, R., LOOMANS, M.E., JANUSZ, J.M., BOHNE, R.L., MADDIN, C.S., GARDNER, J., LAHANN, T.R., FARMER, R.W., JONES, L., CHIABRANDO, C. AND FANELLI, R. (1987) NE-19550: a novel, orally active anti-inflammatory analgesic. *Drugs Exptl. Clin. Res.* **13**: 259-265.

BROCK, J.W. AND JOSHUA, I.G. (1991) Substance P mechanisms in the regulation of striated muscle microcirculation. *Microvasc. Res.* **41**: 24-28.

BUCK, S.H. AND BURKS, T.F. (1986) The neuropharmacology of capsaicin: review of some recent observations. *Pharmacol. Rev.* **38**: 179-226.

BURKS, T.F. AND COOPER, T. (1967) Enhancement of peripheral alpha-receptor stimulation by blockade of "silent" beta receptors. *Circ. Res.* **21**: 703-715.

CAMBRIDGE, H. AND BRAIN, S.D. (1992) Calcitonin gene-related peptide increases blood flow and potentiates plasma protein extravasation in the rat knee joint. *Br. J. Pharmacol.* **106**: 746-750.

CAMERON-SMITH, D., COLQUHOUN, E.Q., YE, J.-M., HETTIARACHCHI, M. AND CLARK, M.G. (1990) Capsaicin and dihydrocapsaicin stimulate oxygen consumption in the perfused rat hindlimb. *Int. J. Obesity* **14**: 259-270.

CAMPBELL, E., BEVAN, S. AND DRAY, A. (1993) Clinical applications of capsaicin and its analogues. In: *Capsaicin in the Study of Pain.* J.N. Wood (ed.), pp. 255-273, Academic Press Ltd., London.

CASINI, A., GALLI, G., SALZANO, R., SANTICIOLI, P., MAGGI, C.A., ROTELLA, C.M. AND SURRENTI, C. (1991) Calcitonin gene-related peptide increases the production of glycoaminoglycans but not collagen type I and III in cultures of rat fat-storing cells. *Life Sci.* **49**: PL163-PL168.

CASINO, P.R., KILCOYNE, C.M., CANNON, R.O. III, QUYYUMI, A.A., AND PANZA, J.A. (1995) Impaired endothelium-dependent vascular relaxation in patients with hypercholesrolemia extends beyond the muscarinic receptor. *Am. J. Cardiol.* **75**: 40-44.

CATERINA, M.J., SCHUMACHER, M.A., TOMINAGA, M., ROSEN, T.A., LEVINE, J.D., AND JULIUS, D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**: 816-824.

CHAHL, L.A. (1989) The effects of ruthenium red on the response of the guinea-pig ilium to capsaicin. *Eur. J. Pharmacol.* **169**: 241-247.

CHAHL, L.A. AND LYNCH, A.M. (1987) The acute effects of capsaicin on the cardiovascular system. *Acta Physiol. Hung.* **69**: 413-419.

CHIBA, T., YAMAGUCHI, A., YAMATANI, T., NAKAMURA, A., MORISHITA, T., INUI, T., FUKAASI, M., NODA, T AND FUJITA, T. (1989) Calcitonin gene-realted peptide antagonist human CGRP-(8-37). *Am. J. Physiol.* **256**: E331-E335.

CHRISTOFORIDES, C., LAASBERG, L.H. AND HEDLEY-WHYTE, J. (1969) Effect of temperature on solubility of O_2 in human plasma. *J. Appl. Physiol.* **26**: 56-60.

CHUNG, F.Z., WU, L.H., VARTANIAN, M.A., WATLING, K.J., GUARD, S., WOODRUFF, G.N. AND OXENDER, D.L. (1994) The non-peptide tachykinin NK2 receptor antagonist SR 48968 interacts with human, but not rat, cloned tachykinin NK3 receptors. *Biochem. Biophys. Res. Comm.* **198**: 967-972.

CLAPHAM, D.E. (1997) Some like it hot: spicing up ion channels. *Nature* **389**: 783-784.

CLARK, M.G., COLQUHOUN, E.Q., RATTIGAN, S., DORA, K.A., ELDERSHAW, T.P.D., HALL, J.L. AND YE, J. (1995) Vascular and endocrine control of muscle metabolism. *Am. J. Physiol.* **268**: E797-E812.

CLARK, M.G., RATTIGAN, S., DORA, K.A., NEWMAN, J.M.B., STEEN, J.T., MILLER, K.A., AND VINCENT, M.A. (1997) Vascular and metabolic regulation of muscle. In: *Physiology, Stress and Malnutrition: Functional Correlates, Nutritional Intervention.* J.M. Kinney and H.N. Tucker (editors), pp Lippincott-Raven Publishers, New York.

COCKCROFT, J.R., CHOWIENCZYK, P.J., BRETT, S.E., AND RITTER, J.M. (1994) Effect of N^G-monomethyl-L-arginine on kinin-induced vasodilatation in the human forearm. *Br. J. Clin. Pharmacol.* **38**: 307-310.

COLQUHOUN, E.Q. AND CLARK, M.G. (1991) Open question: has thermogenesis in muscle been overlooked and misinterpreted? *News Physiol. Sci.* **6**: 256-259.

COLQUHOUN, E.Q., HETTIARACHCHI, M., YE, J-M., RICHTER, E.A., HNIAT, A.J., RATTIGAN, S. AND CLARK, M.G. (1988) Vasopressin and angiotensin II stimulate oxygen uptake in the perfused rat hindlimb. *Life Sci.* **43**: 1747-1754.

COLQUHOUN, E.Q., HETTIARACHCHI, M., YE, J-M., RATTIGAN, S. AND CLARK, M.G. (1990) Inhibition by vasodilators of noradrenalin and vasoconstrictor-mediated, but not skeletal muscle contraction-induced oxygen uptake in the perfused rat hindlimb: implications for non-shivering thermogenesis in muscle tissue. *Gen. Pharmacol.* **21**: 141-148.

COLQUHOUN, E.Q., ELDERSHAW, T.P.D., BENNETT, K.L., HALL, J.L., DORA, K.A. AND CLARK, M.G. (1995) Functional and metabolic evidence for two different vanilloid (VN_1 and VN_2) receptors in perfused rat hindlimb. *Life Sci.* **57**: 91-102.

CÔTÉ, C., THIBAULT, M.C., AND VALLIÈRES, J. (1985) Effect of endurance training and isoproterenol treatment on skeletal muscle sensitivity to norepinephrine. *Life Sci.* **37**: 695-701.

COUTURE, R., LANEUVILLE, O., GUIMOND, C., DRAPEAU, G AND REGOLI, D. (1989) Characterisation of the peripheral action of neurokinins and neurokinin receptor selective agonists on the rat cardiovascular system. *Naunyn-Scmiedeberg's Arch. Pharmacol.* **340**: 547-557.

CRAFT, R.M. AND PORRECA, F. (1992) Treatment parameters of desensitisation to capsaicin. *Life Sci.* **51**: 1767-1775.

CRAYTON, S.C., MITCHELL, J.H. AND PAYNE III, F.C. (1981) Reflex cardiovascular response during injection of capsaicin into skeletal muscle. *Am. J. Physiol.* **240**: H315-H319.

CROCI, T., EMONDS-ALT, X., LE FUR, G. AND MANARA, L. (1995) *In vitro* characterisation of the non-peptide tachykinin NK1 and NK2 receptor antagonists, SR 140333 and SR 48968, in different rat and guinea-pig intestinal segments. *Life Sci.* **56**: 267-275.

CUI, J. AND HIMMS-HAGEN, J. (1992a) Rapid but transient atrophy of brown adipose tissue in capsaicin-desensitised rats. *Am. J. Physiol.* **262** (*Regulatory Integrative Comp. Physiol.* **31**): R562-R567.

CUI, J. AND HIMMS-HAGEN, J. (1992b) Long-term decrease in body fat and in brown adipose tissue in capsaicin-desensitised rats. *Am. J. Physiol.* **262** (*Regulatory Integrative Comp. Physiol.* **31**): R568-R573.

DAVIS, A.J. AND PERKINS, M.N. (1996) Substance P and capsaicin-induced mechanical hyperalgesia in the rat knee joint – the involvement of bradykinin B1 and B2 receptors. *Br. J. Pharmacol.* **118**: 2206-2212.

DELAY-GOYET, P., SATOH, H., AND LUNDBERG, J.M. (1992) Relative involvement of substance P and CGRP mechanisms in antidromic vasodilatation in the rat skin. *Acta Physiol. Scand.* **146**: 537-538.

DENNIS, T. FOURNIER, A., CADIEUX, A., POMERLEAU, F., JOLICOEUR, F.B., ST. PIERRE, S AND QUIRION, R. (1990) $hCGRP_{(8-37)}$, a calcitonin gene-related peptide antagonist revealing calcitonin gene-related peptide receptor heterogeneity in brain and periphery. *J. Pharmacol. Exp. Ther.* **254**: 123-128.

DE VRIES, D.J. AND BLUMBERG, P.M. (1989) Thermoregulatory effects of resiniferatoxin in the mouse: comparison with capsaicin. *Life Sci.* **44**: 711-714.

DICKENSON, A.H. AND DRAY, A. (1991) Selective antagonism of capsaicin by capsazepine: evidence for a spinal receptor site in capsaicin-induced antinociception. *Br. J. Pharmacol.* **104**: 1045-1049.

DIDION, S.P. AND MAYAN, W.G. (1997) Effect of chronic myocardial infarction on *in vivo* reactivity of skeletal muscle arterioles. *Am. J. Physiol.* **272** (*Heart Circ. Physiol. 41*): H2403-H2408.

DOCHERTY, R.J., YEATS, J.C. AND PIPER, A.S. (1997) Capsazepine block of voltage-activated calcium channels in adult rat dorsal root ganglion neurons in culture. *Br. J. Pharmacol.* **121**: 1461-1467.

DONNERER, J. AND LEMBECK, F. (1982) Analysis of the effects of intravenously injected capsaicin in the rat. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **320**: 54-57.

DONNERER, J. AND LEMBECK, F. (1983) Capsaicin-induced reflex fall in rat blood pressure is mediated by afferent substance P-containing neurons via a reflex centre in the brain stem. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **324**: 293-295.

DONNERER, J., AMANN, R., SCHULIGOI, R. AND LEMBECK, F. (1990) Absorption and metabolism of capsaicinoids following intragastric administration in rats. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **342**: 357-361.

DORA, K.A., COLQUHOUN, E.Q., HETTIARACHCHI, M., RATTIGAN, S. AND CLARK, M.G. (1991) The apparent absence of serotonin-mediated vascular thermogenesis in perfused rat hindlimb may result from vascular shunting. *Life Sci.* **48**: 1555-1564.

DORA, K.A., RICHARDS, S.M., RATTIGAN, S., COLQUHOUN, E.Q., AND CLARK, M.G. (1992) Serotonin and norepinephrine vasoconstriction in rat hindlimb have different oxygen requirements. *Am. J. Physiol.* **262**: H698-H703.

DORA, K.A., RATTIGAN, S., COLQUHOUN, E.Q., AND CLARK, M.G. (1994) Aerobic muscle contraction impaired by serotonin-mediated vasoconstriction. *J. Appl. Physiol.* **77**: 277-284.

D'ORLÉANS-JUSTE, P., DION, P., DRAPEAU, G., AND REGOLI, D. (1985) Different receptors are involved in the endothelium-mediated relaxation and the smooth muscle contraction of the rabbit pulmonary artery in response to substance P and related neurokinins. *Eur. J. Pharmacol.* **125**: 37-44.

DOYLE, M.P. AND DULING, B.R. (1997) Acetylcholine induces conducted vasodilatation by nitric oxide-dependent and –independent mechanisms. *Am. J. Physiol.* **272**: H1364-H1371.

DRAY, A. (1992) Mechanism of action of capsaicin-like molecules on sensory neurons. *Life Sci.* **51**: 1759-1765.

DRAY, A. AND URBAN, L. (1996) New pharmacological strategies for pain relief. *Ann Rev. Pharmacol. Toxicol.* **36**: 253-280.

DRAY, A., FORBES, C.A., AND BURGESS, G.M. (1990a) Ruthenium red blocks the capsaicin-induced increase in intracellular calcium and activation of membrane currents in sensory neurons as well as the activation of peripheral nociceptors *in vitro*. *Neurosci. Lett.* **110**: 52-59.

DRAY, A., BETTANY, J. AND FORSTER, P. (1990b) Actions of capsaicin on peripheral nociceptors of the rat spinal cord-tail *in vitro*: dependence of extracellular ions and independence of second messengers. *Br. J. Pharmacol.* **101**: 727-733.

DRAY, A., CAMPBELL, E.A., HUGHES, G.A., PATEL, I.A., PERKINS, M.N., RANG, H.P., RUEFF, A., SENO, N., URBAN, L. AND WALPOLE, C.S.J. (1991) Antagonism of capsaicin-induced activation of C-fibres by a selective capsaicin antagonist, capsazepine. *Br. J. Pharmacol.* **99**: 78P.

DUBOIS, J.M. (1982) Capsaicin blocks one class of K^+ channels in the frog node of Ranvier. *Brain Res.* **245**: 372-375.

DUCKLES, S.P. (1986) Effects of capsaicin on vascular smooth muscle. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **333**: 59-64.

EDVINSSON, L., JANSEN, I., KINGMAN, T.A., MCCULLOCH, J. (1990) Cerebrovascular responses to capsaicin *in vitro* and *in situ*. *Br. J. Pharmacol.* **100**: 312-318.

EDWARDS, S.J., MONTGOMERY, I.M., COLQUHOUN, E.Q., JORDAN, J.E. AND CLARK, M.G. (1992) Spicy meal disturbs sleep: an effect on thermoregulation? *Int. J. Psychophysiol.* **13**: 97-100.

ELDERSHAW, T.P.D. (1996) *Vascular involvement in skeletal muscle metabolism.* PhD. Thesis, Division of Biochemistry, University of Tasmania, Australia.

ELDERSHAW, T.P.D., COLQUHOUN, E.Q., DORA, K.A., PENG, Z.-C. AND CLARK, M.G. (1992) Pungent principles of ginger (*Zingiber officinale*) are thermogenic in the perfused rat hindlimb: *Int. J. Obesity* **16**: 755-763.

ELDERSHAW, T.P.D., DORA, K.A., BENNETT, K., CLARK, M.G. AND COLQUHOUN, E.Q. (1994) Resiniferatoxin and piperine: capsaicin-like stimulators of oxygen uptake in the perfused rat hindlimb. *Life Sci.* **55**: 389-397.

ELLIS, J.L. AND UNDEM, B.J. (1994) Inhibition by capsazepine of resiniferatoxin- and capsaicin induced contractions of guinea pig trachea. *J. Pharmacol. Exp. Ther.* **268**: 85-89.

EMONDS-ALT, X., VILAIN, P., GOULAOUIC, P., PROIETTO, V., VAN BROEK, D., ADVENIER, C., NALINE, E., NELIAT, G., LE FUR, G., AND BRELIÈRE, J.C. (1992) A potent selective non-peptide antagonist of the neurokinin A (NK2) receptor. *Life Sci.* **50**: PL101-PL106.

EMONDS-ALT, X., BICHON, D., DUCOUX, J.P., HEAULME, M., MILOUX, B., PONCELET, M., PROIETTO, V., VAN BROEK, D., VILAIN, P., NELIAT, G., SOUBRIE, P., LE FUR, G., AND BRELIERE, J-C. (1994) SR 142801, the first potent non-peptide antagonist of the tachykinin NK3 receptor. *Life Sci.* **56**: PL27-PL32.

FARKAS-SZALLASI, T., BENNETT, G.J., BLUMBERG, P.M., HÖKFELT, T., LUNDBERG, J.M. AND SZALLASI, A. (1996) Vanilloid receptor loss is independent of the messenger plasticity that follows systemic resiniferatoxin administration. *Brain Res.* **719**: 213-218.

FERREL, W.R., MCDOUGALL, J.J. AND BRAY, R.C. (1997) Spatial heterogeneity of the effects of calcitonin gene-related peptide (CGRP) on the microvascular of ligaments of the rabbit knee joint. *Br. J. Pharmacol.* **121**: 1397-1405.

FIGINI, M., EMANUELI, C., BERTRAND, C., JAVDAN, P. AND GEPPETTI, P. (1996) Evidence that tachykinins relax the guinea-pig trachea via nitric oxide release and by stimulation of a septide-insensitive NK1 receptor. *Br. J. Pharmacol.* **117**: 1270-1276.

FOULKES, R., SHAW, N., BOSE, C. AND HUGHES, B. (1991) Differential vasodilator profile of calcitonin gene-related peptide in porcine large and small diameter coronary artery rings. *Eur. J. Pharmacol.* **201**: 143-149.

FRANCO-CERECEDA, A. AND LUNDBERG, J.M. (1988) Actions of calcitonin gene-related peptide and tachykinins in relation to the contractile effects of capsaicin in the guinea-pig and rat heart *in vitro*. *Naunyn Schmiedeberg's Arch. Pharmacol.* **337**: 649-655.

FRANCO-CERECEDA, A. AND LUNDBERG, J.M. (1992) Capsazepine inhibits low pH- and lactic acid-evoked release of calcitonin gene-related peptide from sensory nerves in guinea-pig heart. *Eur. J. Pharmacol.* **221**: 183-184.

FRANCO-CERECEDA, A., KALLNER, G. AND LUNDBERG, J.M. (1993) Capsazepine-sensitive release of calcitonin gene-related peptide from C-fibre afferents in the guinea-pig heart by low pH and lactic acid. *Eur. J. Pharmacol.* **238**: 311-316.

GARRET, C., CARRUETTE, A., FARDIN, V., MOUSSAOUI, S., PEYRONEL, J-F., BLANCHARD, J-C., AND LADURON, P.M. (1991) Pharmacological properties of a potent and selective non-peptide substance P antagonist. *Proc. Natl. Acad. Sci. USA* **88**: 10208-10212.

GIULIANI, S., TURINI, D., BARBANTI, G. AND MAGGI, C.A. (1991) Ruthenium red as a selective capsaicin antagonist of the motor response to capsaicin in the human isolated ileum. *Eur. J. Pharmacol.* **196**: 331-333.

GIULIANI, S., WIMALAWANSA, S.J., AND MAGGI, C.A. (1992) Involvement of multiple receptors in the biological effects of calcitonin gene-related peptide and amylin in rat guineas-pig preparations. *Br. J. Pharmacol.* **107**: 510-514.

GOSO, C., MANZINI, S., BLUMBERG, P.M., AND SZALLASI, A. (1993a) Competitive inhibition by capsazepine of [^{3}H]-resiniferatoxin binding to the vanilloid receptor. *Br. J. Pharmacol.* **108** (Suppl.): 306P.

GOSO, C., EVANGELISTA, S., TRAMONTANA, M., MANZINI, S., BLUMBERG, P.M., AND SZALLASI, A. (1993b) Topical capsaicin administration protects against trinitrobenzene sulfonic acid (TNB)-induced colitis in the rat: characterisation of the vanilloid receptor involved. *Eur. J. Pharmacol.* **249**: 185-190.

GREENBERG, S., LONG, J.P. AND DIECKE, F.P.J. (1973) Differentiation of calcium pools utilised in the contractile response of canine arterial and venous smooth muscle to norepinephrine. *J. Pharmacol. Exp. Ther.* **185**: 493-504.

GRUBB, B. AND FOLK, G.E. (1976) Effect of cold acclimation on norepinephrine induced oxygen consumption in muscle. *J. Comp. Physiol.* **110**: 217-226.

GRUBB, B. AND FOLK, G.E. (1977) The role of adrenoceptors in norepinephrine-stimulated VO_2 in muscle. *Eur.J.Pharmacol.* **43**: 217-223.

GUARD, S., WATSON, S.P., MAGGIO, J.E., TOO, H.P. AND WATLING, K.J. (1990) Pharmacological analysis of [^{3}H]-senktide binding to NK3 tachykinin receptors in guinea-pig longitudinal muscle-myenteric plexus and cerebral cortex membranes. *Br. J. Pharmacol.* **90**: 767-773,.

GUSTAFSSON, L.E., PERSSON, M.G., WEI, S-Z., WICKLAND, N.P. AND ELIAS, Y. (1994) Neurogenic vasodilatation in rabbit hindlimb mediated by tachykinins and nitric oxide. *J. Cardiovasc. Pharmacol.* **23**: 612-617.

HALL, J.M. AND BRAIN, S.D. (1994) Inhibition by SR140333 of NK1 tachykinin receptor-evoked, nitric oxide-dependent vasodilatation in the hamster cheek pouch microvasculature in vivo. *Br. J. Pharmacol.* **113**: 522-526.

HALL, J.L., YE, J-M., CLARK, M.G. AND COLQUHOUN, E.Q. (1997) Sympathetic stimulation elicits increased or decreased VO_2 in the perfused rat hindlimb via α_1-adrenoreceptors. *Am. J. Physiol.* **272**: H2146-H2153.

HAYES, A.G., OXFORD, A., REYNOLDS, M., SHINGLER, A.H., SKINGLE, M., SMITH, C. AND TYERS, M.D. (1984) The effects of a series of capsaicin analogues on nociception and body temperature in the rat. *Life Sci.* **34**: 1241-1248.

HENRY, C.J.K. AND EMERY, B. (1986) Effect of spiced food on metabolic rate. *Human Nutr. Clin. Nutr.* **40C**: 165-168.

HERSHEY, A.D., DYKEMA, P.E. AND KRAUSE, J.E. (1991) Organisation, structure and expression of the gene encoding the rat substance P receptor. *J. Biol. Chem.* **266**: 4366-4374.

HEYMAN, I. AND RANG, H.P. (1985) Depolarising responses to capsaicin in a subpopulation of rat dorsal root ganglion cells. *Neurosci. Lett.* **56**: 69-75.

HIROOKA, Y., IMAIZUMI, T., HARADA, S., MASAKA, H., MOMOHARA, M., TAGAWA, T., AND TAKESHITA, A. (1992) Endothelium-dependent forearm dilatation to

acetylcholine but not substance P is impaired in patients with heart failure. *J. Cardiovasc. Pharmacol.* **20**: S221-225.

HOLZER, P. (1988) Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuoscience* **24**: 739-768.

HOLZER, P. (1991) Capsaicin: cellular targets, mechanisms of action and selectivity for thin sensory neurons. *Pharmacol. Rev.* **42**: 143-201.

HOLZER, P. (1993) Capsaicin-sensitive nerves in the control of vascular effector mechanisms. In: *Capsaicin in the Study of Pain.* J.N. Wood (ed.), pp. 191-218, Academic Press Ltd., London.

HOLZER, P. (1998) Neurogenic vasodilatation and plasma leakage in the skin. *Gen. Pharmacol.* **30**: 5-11.

HOLZER, P. AND JOCIC, M. (1994) Cutaneous vasodilatation induced by nitric oxide-evoked stimulation of afferent nerves in the rat. *Br. J. Pharmacol.* **112**: 1181-1187.

HOLZER, P., LIPPE, I.TH., JOCIC, M., WACHTER, CH., ERB, R. AND HEINMANN, A (1993) Nitric oxide-dependent and –independent hyperaemia due to calcitonin gene-related peptide in the rat stomach. *Br. J. Pharmacol.* **110**: 404-410.

HOLZER, P., WACHTER, CH., HEINMANN, A., JOCIC, M., LIPPE, I.TH. AND HERBERT, M.K. (1995) Diverse interactions of calcitonin gene-related peptide and nitric oxide in the gastric and cutaneous microcirculation. *Can. J. Physiol. Pharmacol.* **73**: 991-994.

HUANG, A. AND KOLLER, A. (1996) Both nitric oxide and prostaglandin-mediated responses are impaired in skeletal muscle arterioles of hypertensive rats. *J. Hypertension* **14**: 887-895.

HUBER, O., BERTRAND, C., BUNNETT, N.W., PELLEGRINI, C.A., NADEL, J.A., NAKAZATO, P., DEBAS, H.T. AND GEPPETTI, P. (1993) Tachykinins mediate the contraction of the human lower esophageal sphincter in vitro via activation of NK2 receptors. *Eur. J. Pharmacol.* **239**: 103-109.

INGI, T., KITAJIMA, Y., MINAMITAKE, Y. AND NAKANISHI, S. (1991) Characterisation of ligand binding properties and selectivities of three rat tachykinin receptors by transfection and functional expression of their cloned cDNAs in mammalian cells. *J. Pharmacol. Exp. Ther.* **259**: 968-975.

JAMES, I.F., NINKINA, N.N. AND WOOD, J.N. (1993) The capsaicin receptor. In: *Capsaicin in the Study of Pain*. J.N. Wood (ed.), pp. 83-102, Academic Press Ltd., London.

KANMURA, Y., RAEYMAEKERS, L. AND CASTEELS, R. (1989) Effects of doxyrubicin and ruthenium red on intracellular Ca^{2+} stores in skinned rabbit mesenteric smooth muscle fibres. *Cell Calcium* **10**: 433-439.

KAUFMAN, M.P., IWAMOTO, G.A., LONGHURST, J.C. AND MITCHELL, J.H. (1982) Effects of capsaicin and bradykinin on afferent fibres with endings in skeletal muscle. *Circ. Res.* **50**: 133-139.

KAWADA, T., WATANABE, T., TAKAISHI, T., TANAKA, T. AND IWAI, K. (1986) Capsaicin-induced β-adrenergic action on energy metabolism in rats: influence of capsaicin on oxygen consumption, the respiratory quotient, and substrate utilisation. *Proc. Soc. Exp. Biol. Med.* **183**: 250-256.

KAWADA, T., SAKABE, S-I., WATANABE, T., YAMAMOTO, M. AND IWAI, K. (1988) Some pungent principles of spices cause the adrenal medulla to secrete catecholamine in anaesthetised rats. *Proc. Soc. Exp. Biol. Med.* **188**: 229-233.

KENAKIN, T. (1993) *Pharmacologic analysis of drug-receptor interaction* (2nd edition), Raven Press Ltd., New York.

KEREZOUDIS, N.P., OLGART, L., AND EDWALL, L. (1993) Differential effects of nitric oxide synthesis inhibition on basal blood flow and antidromic vasodilatation in rat oral tissues. *Eur. J. Pharmacol.* **241**: 209-219.

KIM, C,. ROBERTS, A.M. AND JOSHUA, I.G. (1995) Differences in the capsaicin-induced dilation of arterioles and venules in rat striated muscle. *J. Pharmacol. Exp. Ther.* **273**: 605-610.

KOBINGER, W. AND PITCHLER, L. (1978) Alpha-2 adrenoreceptor agonist effect of B-HT920 in isolated perfused hindquarters of rats. *Eur. J. Pharmacol.* **76**: 101-105.

KREUTTER, D., ORENA, S.J. AND ANDREW, K.M. (1989) Supression of insulin-stimulated glucose transport in L6 myocytes by calcitonin gene-related peptide. *Biochem. Biophys. Res. Commun.* **164**: 461-467.

KUENZI, F.M. AND DALE, N. (1996) Effect of capsaicin and analogues on potassium and calcium currents and vanilloid receptors in Xenopus embryo spinal neurons. *Br. J. Pharmacol.* **119**: 81-90.

KUROIWA, M., AOKI, H., KOBAYASHI, S., NISHIMURA, J., AND KANAIDE, H. (1995) Mechanism of endothelium-dependent relaxation induced by substance P in the coronary artery of the pig. *Br. J. Pharmacol.* **116**: 2040-2047.

LAM, F-Y AND FERRELL, W.R. (1993) Effects of interactions of naturally-occurring neuropeptides on blood flow in the rat knee joint. *Br. J. Pharmacol.* **108**: 694-699.

LAWSON, S.N. AND HARPER, A.A. (1984) Neonatal capsaicin is not a specific neurotoxin for C-fibres or small dark cells in rat dorsal root ganglia. In: *Antidromic Vasodilatation and Neurogenic Inflammation.* Chahl, L.A., Szolcsanyi, J. and Lembeck, F. (eds.), pp. 111-116, Academiai Kiado, Budapest.

LEE, L-Y. AND LUNDBERG, J. (1994) Capsazepine abolishes pulmonary chemoreflexes induced by capsaicin in anaesthetised rats. *J. Appl. Physiol.* **76**: 1848-1855.

LEIGHTON, B. AND FOOT, E.A. (1995) The role of the sensory peptide calcitonin gene-related peptide in skeletal muscle carbohydrate metabolism: effects of capsaicin and resiniferatoxin. *Biochem. J.* **307**: 707-712.

LEMBECK, F. AND HOLZER, P. (1979) Substance P as a neurogenic mediator of antidromic vasodilatation and neurogenic plasma extravasation. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **310**: 175-183.

LEW, R., GERAGHTY, D.P., DRAPEAU, G., REGOLI, D. AND BURCHER, E. (1990) Binding characteristics of [^{125}I]-Bolton-Hunter [Sar^{9}, Met(O$_2$)^{11}] substance P, a new selective radioligand for the NK1 receptor. *Eur. J. Pharmacol.* **184**: 97-108.

LIU, L. AND SIMON, S.A. (1994) A rapid capsaicin-activated current in rat trigeminal ganglion neurons. *Proc. Natl. Acad. Sci.* **91**: 738-741.

LIU, L. AND SIMON, S.A. (1996) Capsaicin-induced currents with distinct desensitisation and Ca2+ dependence in rat trigeminal ganglion neurons. J. Neurophysiol. 75: 1503-1514.

LIU, L., WANG, Y. AND SIMON, S.A. (1996) Capsaicin-activated currents in rat dorsal root ganglion cells. *Pain* **64**: 191-195.

LIU, L. AND SIMON, S.A. (1997) Capsazepine, a vanilloid receptor antagonist, inhibits nicotinic acetylcholine receptors in rat trigeminal ganglia. *Neurosci. Lett.* **228**: 29-32.

LIU, L., SZALLASI, A., AND SIMON, S.A. (1998) A non-pungent resiniferatoxin analogue, phorbol 12-phenylacetate 13-actetate 20-homovanillate, reveals vanilloid receptor subtypes on rat trigemminal ganglion neurons. *Neuroscience* **84**: 569-581.

LOMBET, A. AND SPEDDING, M. (1994) Differential effect of non-peptidic tachykinin receptor antagonists on Ca^{2+} channels. *Eur. J. Pharmacol.* **267**: 113-115.

LOU, Y-P. (1993) Regulation of neuropeptide release from pulmonary capsaicin-sensitive afferents in relation to bronchoconstriction. *Acta Physiol. Scand.* **149** (suppl. 612): 1-88.

LOU, Y-P. AND LUNDBERG, J. (1992) Inhibition of low pH-evoked activation of airway sensory nerves by capsazepine, a novel capsaicin receptor antagonist. *Biochem. Biophys. Res. Comm.* **189**: 537-544.

LOU, Y-P., FRANCO-CERECEDA, A., AND LUNDBERG, J.M. (1992) Different ion channel mechanisms between low concentrations of capsaicin and high concentrations of capsaicin and nicotine regarding peptide release from pulmonary afferents. *Acta Physiol. Scand.* **146**: 119-127.

LOU, Y-P., LEE, L.Y., SATOH, H. AND LUNDBERG, J.M. (1993) Postjunctional inhibitory effect of the NK2 receptor antagonist, SR 48968, on sensory NANC bronchoconstriction in the guinea-pig. *Br. J. Pharmacol.* **109**: 765-773.

LUCOT, J.B., OBACH, R.S., MCLEAN, S. AND WATSON, J.W. (1997) The effect of CP-99,994 on the responses to provocative motion in the cat. *Br. J. Pharmacol.* **120**: 116-120.

LUNDBERG, J. (1993) Capsaicin-sensitive sensory nerves in the airways – implications for protective reflexes and disease. In: *Capsaicin in the Study of Pain.* J.N. Wood (ed.), pp. 219-238, Academic Press Ltd., London.

LUNDBERG, J. (1996) Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. *Pharmacol. Rev.* **48**: 113-178.

McLean, S., Ganong, A., Seymour, P.A., Snider, R.M., Desai, M.C., Rosen, T., Bryce, D.K., Longo, K.P., Reynolds, L.S., Robinson, G., Schmidt, A.W., Siok, C., and Heym, J. (1993) Pharmacology of CP-99,994; a non-peptide antagonist of the tachykinin NK1 receptor. *J Pharmacol. Exp. Ther.* **267**: 472-479.

McLean, S. and Lowe III, J.A. (1994) Agonist and antagonist receptor binding. In: *The Tachykinin Receptors*. S.H. Buck (ed.), pp. 67-99, Humana Press Inc., Totowa, NJ, USA.

McMurdo, L., Lockhart, J.C. and Ferrell, W.R. (1997) Modulation of synovial blood flow by the calcitonin gene-related peptide (CGRP) recepttor antagonist, $CGRP_{(8-37)}$. *Br. J. Pharmacol.* **121**: 1075-1080.

Maggi, C.A. (1993) The pharmacological modulation of neurotransmitter release. In: *Capsaicin in the Study of Pain*. J.N. Wood (ed.), pp. 161-190, Academic Press Ltd., London.

Maggi, C.A. (1992) Therapeutic potential of capsaicin-like molecules: studies in animals and humans. *Life Sci.* **51**: 1777-1781.

Maggi, C.A. (1995) The mammalian tachykinin receptors. *Gen. Pharmacol.* **26**: 911-944.

Maggi, C.A. and Giuliani, S. (1996) Evidence that tachykinin NK2 receptors modulate resting tone in the rat isolated small intestine. *Br. J. Pharmacol.* **118**: 1262-1268.

Maggi, C.A. and Meli, A. (1988) The sensory efferent function of capsaicin-sensitive sensory neurons. *Gen. Pharmac.* **19**: 1-43.

Maggi, C.A. and Schwartz, T.W. (1997) The dual nature of the tachykinin NK1 receptor. *Trends Pharmacol. Sci.* **18**: 351-354.

MAGGI, C.A., PATTACHINI, R., SANTICIOLI, P., LIPPE, I. TH., GIULIANI, S., GEPPETTI, P., DEL BIANCO, E., SELLERI, S. AND MELI, A. (1988a) The effect of omega conotoxin GVIA, a peptide modulator of N-type voltage-sensitive calcium channels, on motor responses produced by activation of efferent and sensory nerves in mammalian smooth muscle. *Naunyn Schmiedeberg's Arch. Pharmacol.* **338**: 107-113.

MAGGI, C.A., PATTACHINI, R., SANTICIOLI, P., GIULIANI, S., GEPPETTI, P. AND MELI, A. (1988b) Protective action of ruthenium red toward capsaicin desensitisation of sensory fibres. *Neurosci. Lett.* **88**: 201-205.

MAGGI, C.A., SANTICIOLI, P., GEPPETTI, P., PARLANI, M., ASTOLFI, M., DEL BIANCO, E., PATTACHINI, R., SELLERI, S., GIULIANI, S. AND MELI, A. (1989) The effect of calcium-free medium and nifedipine on the release of substance P-like immunoreactivity and contractions induced by capsaicin in the isolated guinea-pig and rat bladder. *Gen. Pharmacol.* **20**: 445-456.

MAGGI, C.A., BEVAN, S., WALPOLE, C.S.J., RANG, H.P., AND GIULIANI, S. (1993a) A comparison of capsazepine and ruthenium red as capsaicin antagonists in the rat isolated urinary bladder and vas deferens. *Br. J. Pharmacol.* **108**: 801-805.

MAGGI, C.A., PATACCHINI, R., ROVERO, P. AND GIACHETTI, A. (1993b) Tachykinin receptors and tachykinin receptor antagonists. *J. Auton. Pharmacol.* **13**: 23-93.

MAGGI, C.A., PATACCHINI, R., GIULIANI, S., AND GIACHETTI, A. (1993c) In vivo and in vitro pharmacology of SR 48968, a non-peptide tachykinin NK2 receptor antagonist. *Eur J. Pharmacol.* **234**: 83-90.

MALCANGIO, M. AND BOWERY, N.G. (1994) Effect of the tachykinin NK1 receptor antagonists, RP 67580 and SR140333, on electrically-evoked substance P release from rat spinal cord. *Br. J. Pharmacol.* **113**: 635-641.

MARSH, S.J., STANSFELD, C.E., BROWN, D.A., DAVEY, R. AND MCCARTHY, D. (1987) The mechanism of action of capsaicin on C-type neurons and their axons *in vitro*. *Neuroscience* **23**: 275-289.

MARSHALL, I. (1992) Mechanism of vascular relaxation by calcitonin gene-related peptide. *Ann. N.Y. Acad. Sci.* **657**: 204-215.

MARTIN, C.A.E., EMONDS-ALT, X., AND ADVENIER, C. (1993) Inhibition of cholinergic neurotransmission in isolated guinea-pig main bronchi by SR 48968. *Eur. J. Pharmacol.* **243**: 309-312.

MASTRANGELO, D., MATHISON, R., HUGGEL, H.J., DION, S., D'ORLEANS-JUSTE, P., RHALEB, N.-E., DRAPEAU, G., ROVERO, P. AND REGOLI, D. (1987) The rat isolated portal vein: a preparation sensitive to neurokinins, particularly neurokinin B. *Eur. J. Pharmacol.* **134**: 321-326.

MASU, Y., NAKAYAMA, K., TAMAKI, H., HARADA, Y., KUNO, M. AND NAKANISHI, S. (1987) cDNA cloning of substance K receptor through oocyte expression system. *Nature* **329**: 836-838.

MILLER, M.S., BUCK, S.H., SIPES, I.G. AND BURKS, T.F. (1982a) Capsaicinoid-induced local and systemic antinociception without substance P depletion. *Brain Res.* **244**: 193-197.

MILLER, M.S., BUCK, S.H., SIPES, I.G. YAMAMURA, H.I. AND BURKS, T.F. (1982b) Regulation of substance P by nerve growth factor: disruption by capsaicin. *Brain Res.* **250**: 193-196.

MOORE, C.L. (1971) Specific inhibition of mitochondrial Ca^{2+} transport by ruthenium red. *Biochem. Biophys. Res. Comm.* **42**: 298-305.

MOORE, B.A., VANNER, S., BUNNETT, N.W. AND SHARKEY, K.A. (1997) Characterisation of neurokinin 1 receptors in the submucosal plexus of guinea-pig ileum. *Am. J. Physiol.* **272**: G670-G678.

MUSSAP, C.J., LEW, R. AND BURCHER, E. (1989) The autoradiographic distribution of substance P binding sites in the guinea-pig *vas deferens* is altered by capsaicin pretreatment. *Eur. J. Pharmacol.* **168**: 337-345.

MUSSAP, C.J., GERAGHTY, D.P. AND BURCHER, E. (1993) Tachykinin receptors: a radioligand binding perspective. *J. Neurochem.* **60**: 1987-2009.

NAKANISHI, S., NAKAJIMA, Y. AND YOKOTA, Y. (1993) Signal transduction and ligand binding domains of the tachykinin receptors. *Regul. Peptides* **46**: 37-42.

NANTEL, F., ROUISSI, N., RHALEB, N-E., DION, S., DRAPEAU, G., AND REGOLI, D. (1990) The rabbit jugular vein is a contractile NK-1 receptor system. *Eur. J. Pharmacol.* **179**: 457-462.

NEWBY, D.E., BOON, N.A., AND WEBB, D.J. (1997a) Comparison of forearm vasodilatation to substance P and acetylcholine: contribution of nitric oxide. *Clin. Sci.* **92**: 133-138.

NEWBY, D.E., SCIBERRAS, D.G., MENDEL, C.M., GERTZ, B.J., BOON, N.A., AND WEBB, D.J. (1997b) Intra-arterial substance PO mediated vasodilatation in the human forearm: pharmacology, reproducibility and tolerability. *Br. J. Clin. Pharacol.* **43**: 493-499.

NEWMAN, J.M.B., DORA, K.A., RATTIGAN, S., EDWARDS, S.J., COLQUHOUN, E.Q. AND CLARK, M.G. (1996) Norepinephrine and serotonin vasoconstriction in rat hindlimb control different vascular flow routes. *Am. J. Physiol.* **270**: E689-E699.

NEWMAN, J.M.B., STEEN, J.T. AND CLARK, M.G. (1997) Vessels supplying septa and tendons as functional shunts in perfused rat hindlimb. *Microvasc. Res.* **54**: 49-57.

NEWMAN, J.M.B. AND CLARK, M.G. (1998) Stimulation and inhibition of resting muscle thermogenesis by vasoconstrictors in perfused rat hindlimb. *Can. J. Physiol. Pharmacol.* In press.

OTSUKA, M. AND YOSHIOKA, K. (1993) Neurotransmitter functions of mammalian tachykinins. *Physiol. Rev.* **73**: 229-308.

PELLETIER, C.L. AND SHEPHERD, J.T. (1975) Relative influence of carotid baroreceptors and muscle receptors in the control of renal and hindlimb circulations. *Can. J. Physiol. Pharmacol.* **53**: 1042-1049.

PANZA, J.A., CASINO, P.R., KILCOYNE, C.M., AND QUYYUMI, A.A. (1994) Impaired endothelium-dependent vasodilatation in patients with essential hypertension: evidence that abnormality is not at the muscarinic receptor level. *J. Am. Coll. Cardiol.* **23**: 1610-1616.

PARLANI, M., CONTE, B., GOSO, C., SZALLASI, A., AND MANZINI, S. (1993) Capsaicin-induced relaxation in the external urethral sphincter: characterisation of the vanilloid receptor and mediation by CGRP. *Br. J. Pharmacol.* **110**: 989-994.

PELLETIER, C.L. AND SHEPHERD, J.T. (1975) Relative influence of carotid baroreceptors in the control of renal and hindlimb circulations. *Can. J. Physiol. Pharmacol.* **53**: 1042-1049.

PERSSON, M.G., HEDQVIST, P. AND GUSTAFSSON, L.E. (1991) Nerve-induced tachykinin-mediated vasodilatation in skeletal muscle is dependent on nitric oxide formation. *Eur. J. Pharmacol.* **205**: 295-301.

PITTNER, R.A., WOLFE-LOPEZ, D., YOUNG, A.A. AND BEAUMONT, K. (1996) Different pharmacological characteristics in L6 and C_2C_{12} muscle cells and intact skeletal muscle for amylin, CGRP and calcitonin. *Br. J. Pharmacol.* **117**: 847-852.

PLOUG, T., GALBO, H., VINTEN, J., JORGENSEN, M. AND RICHTER, E.A. (1987) Kinetics of glucose transport in rat muscle: effects of insulin and contractions. *Am. J. Physiol.* **253**: E12-E20.

POPPER, P. AND MYCEVYCH, P.E. (1989) Localisation of calcitonin gene-related peptide and its receptors in a striated muscle. *Brain Res.* **496**: 180-186.

POYNER, D. (1992) Calcitonin gene-related peptide: multiple actions, multiple receptors. *Pharmac. Ther.* **56**: 23-51.

POYNER, D. (1995) Pharmacology of receptors for calcitonin gene-related peptide and amylin. *Trends Pharmacol. Sci.* **16**: 424-428.

POYNER, D., ANDREW, D.P., BROWN, D., BOSE, C. AND HANLEY, M. (1992) Pharmacological characterisation of a receptor for calcitonin gene-related peptide on rat L6 myocytes. *Br. J. Pharmacol.* **105**: 441-447.

QIAN, Y., ADVENIER, C., NALINE, E., BELLAMY, J.F. AND EMONDS-ALT, X. (1994) Effects of SR 48968 on the neuropeptide gamma-induced contraction of the human isolated bronchus. *Fund. Clin. Pharmacol.* **8**: 71-75.

RALEVIC, V., KHALIL, Z., DUSTING, G.J. AND HELME, R.D. (1992) Nitric oxide and sensory nerves are involved in the vasodilator response to acetylcholine but not calcitonin gene-related peptide in rat skin microvasculature. *Br. J. Pharmacol.* **106**: 650-655.

RATTIGAN, S., DORA, K.A., COLQUHOUN, E.Q. AND CLARK, M.G. (1993) Serotonin-mediated acute insulin resistance in the perfused rat hindlimb but not incubated muscle: a role for the vascular system. *Life Sci.* **53**: 1545-1555.

RATTIGAN, S., DORA, K.A., COLQUHOUN, E.Q. AND CLARK, M.G. (1995) Inhibition of insulin-mediated glucose uptake in hindlimb by an α-adrenergic effect. *Am. J. Physiol.* **268**, E305-E311

RATTIGAN, S., DORA, K.A., TONG, A.C.Y., AND CLARK, M.G. (1996) Perfused skeletal muscle contraction and metabolism improved by angiotensin II-mediated vasoconstriction. *Am. J. Physiol.* **271**: E96-E103.

REES, D.D., PALMER, R.M.J., SCHULTZ, R., HODSON, H.F. AND MONCADA, S. (1990) Characterisation of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br. J. Pharmacol.* **101**: 746-752.

REGOLI, D., DRAPEAU, G., DION, S., D'ORLÉANS-JUSTE, P. (1987) Pharmacological receptors for substance P and neurokinins. *Life Sci.* **40**: 109-117.

REGOLI, D., BOUDON, A., AND FAUCHERE, J-L. (1994) Receptors and antagonists for substance P and related peptides. *Pharmacol. Rev.* **46**: 551-599.

RICHARDS, S.M., DORA, K.A., HETTIARACHICHI, M., RATTIGAN, S., COLQUHOUN, E.Q. AND CLARK, M.G. (1992) A close association between vasoconstrictor-mediated uracil and lactate release by the perfused rat hindlimb. *Gen. Pharmacol.* **23**: 65-69.

RICHTER, E.A., RUDERMAN, N.B. AND GALBO, H. (1982) Alpha and beta adrenergic effects on metabolism in contracting, perfused muscle. *Acta Physiol. Scand.* **116**: 215-222.

RIPPE, B., KAMIYA, A. AND FOLKOW, B. (1978) Simultaneous measurements of capillary diffusion and filtration exchange during shifts in filtration-absorption and at graded alterations in the capillary permeability surface area product (PS). *Acta Physiol. Scand.* **104**: 318-336.

ROVATI, G.E. AND NICOSIA, S. (1994) Lower efficacy: interaction with an inhibitory receptor or partial agonism? *Trends Pharmacol. Sci.* **15**: 140-144.

RUDERMAN, N.B., HOUGHTON, C.S.R. AND HEMS, R. (1971) Evaluation of the isolated perfused rat hindquarter for the study of muscle metabolism. *Biochem. J.* **124**: 639-651.

RUSSELL, J.S., CHI, H., LANTRY, L.E., STEPHENS, R.E. AND WARD, P.E. (1996) Substance P and neurokinin A metabolism by cultured human skeletal muscle myocytes and fibroblasts. *Peptides* **17**: 1397-1403.

SAITO, A., MASAKI, T., LEE, T.J.-F. AND GOTO, K. (1988) Effects of capsaicin on the contractility and peptide-containing nerves of large cerebral arteries of the cat. *Arch. Int. Pharmacodyn.* **295**: 194-203.

SALVIATI, G. AND VOLPE, P. (1988) Ca^{2+} release from sarcoplasmic reticulum of skinned fast- and slow-twitch muscle fibres. *Am. J. Physiol.* **254**: C459-C465.

SANTICIOLI, P., DEL BLANCO, E., GEPPETTI, P. AND MAGGI, C.A. (1992) Release of calcitonin gene-related peptide-like (CGRP-LI) immunoreactivity from rat isolated soleus muscle by low pH, capsaicin and potassium. *Neurosci. Lett.* **143**: 19-22.

SANTICIOLI, P., DEL BLANCO, E., FIGINI, M., BEVAN, S.. AND MAGGI, C.A. (1993) Effect of capsazepine on the release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) induced by low pH, capsaicin and potassium in rat soleus muscle. *Br. J. Pharmacol.* **110**: 609-612.

SATOH, T., MIYOSHI, H., SAKAMOTO, K., IWAMURA, H. (1996) Comparison of the inhibitory action of capsaicin analogues with various NADH-ubiquinone oxidoreductases. *Biochim. Biophys. Acta* **1273**: 21-30.

SENO, N. AND DRAY, A. (1993) Capsaicin-induced activation of fine afferent fibres from rat skin in vitro. *Neuroscience* **55**: 563-569.

SHIGEMOTO, R., YOKOTA, Y., TSUCHIDA, K. AND NAKANISHI, S. (1990) Cloning and expression of a rat neuromedin K receptor cDNA. *J. Biol. Chem.* **265**: 623-628.

SHIMOMURA, Y., KAWADA, T. AND SUZUKI, M. (1989) Capsaicin and its analogues inhibit the activity of NADH-coenzyme Q oxidoreductase of the mitochondrial respiratory chain. *Arch. Biochem. Biophys.* **270**: 573-577.

SHIRAHASE, H., KANDA, M., KURAHASHI, K., NAKAMURA, S., USUI, H. AND SHIMIZU, Y. (1995) Endothelium-dependent contraction in intrapulmonary arteries: mediation by endothelial NK1 receptors and TXA_2. *Br. J. Pharmacol.* **115**: 1215-1220.

SHIRAMOTO, M. AND IMAIZUMI, T., (1997) Contribution of nitric oxide to vasodilatation by substance P in human forearm vessels: authors reply. *Clin. Sci.* **92**: 607-608.

SHIRAMOTO, M., IMAIZUMI, T., HIROOKA, Y., ENDO, T., NAMBA, T., OYAMA, J-I., HIRONAGA, K., AND TAKESHITA, A. (1997) Role of nitric oxide towards vasodilator effects of substance P and ATP in human forearm vessels. *Clin. Sci.* **92**: 123-131.

SIETSEMA, W.K., BERMAN, E.F., FARMER, R.W. AND MADDIN, C.S. (1988) The antinociceptive effect and pharmacokinetics of olvanil following subcutaneous dosing in the mouse. *Life Sci.* **43**: 1385-1391.

SIRÉN, A-L. AND FEUERSTEIN, G. (1988) Cardiovascular effects of rat calcitonin gene-related peptide in the conscious rat. *J. Pharmacol. Exp. Ther.* **247**: 69-78.

SNIDER, R.M., CONSTANTINE, J.W., LOWE III, J.A., LONGO, K.P., LEBEL, W.S., WOODY, H.A., DROZDA, S.E., DESAI, M.C., VINICK, F.J., SPENCER, R.W., HESS, H-J. (1991) A potent non-peptide antagonist of the substance P (NK1) receptor. *Science* **251**: 435-437.

STEWART-LEE, A. AND BURNSTOCK, G. (1989) Actions of tachykinins on the rabbit mesenteric artery: substance P and [Glp^6, L-Pro^9]SP_{6-11} are potent agonists for endothelial neurokinin-1 receptors. *Br. J. Pharmacol.* **97**: 1218-1224.

SZABADI, E. (1977) A model of two functionally antagonistic receptor populationsactivated by the same agonist. *J. Theor. Biol.* **69**: 101-112.

SZALLASI, A. (1994) The vanilloid (capsaicin) receptor: receptor types and species differences. *Gen Pharmac.* **25**: 223-243.

SZALLASI, A. (1995) Autoradiographic visualisation and pharmacological characterisation of vanilloid (capsaicin) receptors in several species, including man. *Acta Physiol. Scand.* **155** (Suppl. 629): 1-68.

SZALLASI, A. AND BLUMBERG, P.M. (1989) Resiniferatoxin, a phorbol-related diterpene, acts as an ultrapotent analogue of capsaicin, the irritant constituent in red pepper. *Neuroscience* **30**:515-520.

SZALLASI, A. AND BLUMBERG, P.M. (1990a) Specific binding of resiniferatoxin, an ultrapotent capsaicin analog, by dorsal root ganglion membranes. *Brain Res.* **524**: 106-111.

SZALLASI, A. AND BLUMBERG, P.M. (1990b) Resiniferatoxin and its analogs provide novel insights into the pharmacology of the vanilloid (capsaicin) receptor. *Life Sci.* **47**: 1399-1408.

SZALLASI, A. AND BLUMBERG, P.M. (1991) Molecular target size of the vanilloid (capsaicin) receptor in pig dorsal root ganglia. *Life Sci.* **48**: 1863-1869.

SZALLASI, A. AND BLUMBERG, P.M. (1992) Vanilloid receptor loss in rat sensory ganglia associated with long-term desensitisation to resiniferatoxin. *Neurosci. Lett.* **136**: 51-54.

SZALLASI, A. AND BLUMBERG, P.M. (1993) [^{3}H]-resiniferatoxin binding by the vanilloid receptor: species-related differences, effects of temperature and sulfhydryl reagents. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **347**: 84-91.

SZALLASI, A. AND BLUMBERG, P.M. (1996) Vanilloid receptors; new insights enhance potential as a therapeutic target. *Pain* **68**: 195-208.

SZALLASI, A., LEWIN, N.E. AND BLUMBERG, P.M. (1992) Identification of α_1-acid glycoprotein (orosomucoid) as a major vanilloid binding protein in serum. *J. Pharmacol. Exp. Ther.* **262**: 883-888.

SZALLASI, A., CONTE, B., GOSO, C., BLUMBERG, P.M., AND MANZINI, S. (1993a) Characterisation of a peripheral vanilloid (capsaicin) receptor in the urinary bladder of the rat. *Life Sci.* **52**: PL221-226.

SZALLASI, A., LEWIN, N.A. AND BLUMBERG, P.M. (1993b) Vanilloid (capsaicin) receptor in the rat: positive cooperativity of resiniferatoxin binding and its modulation by reduction and oxidation. *J. Pharmacol. Exp. Ther.* **266**: 678-683.

SZALLASI, A., GOSO, C., BLUMBERG, P.M. AND MANZINI, S. (1993c) Competitive inhibition by capsazepine of [^{3}H]-resiniferatoxin binding to central (spinal cord and dorsal root ganglia) and peripheral (urinary bladder and airways) vanilloid (capsaicin) receptors in the rat. *J. Pharmacol. Exp. Ther.* **267**: 728-733.

SZALLASI, A., NILSSON, S., HÖKFELT, T. AND LUNDBERG, J.M., (1994) Visualising vanilloid (capsaicin) receptors in pig spinal cord by [^{3}H]-resiniferatoxin autoradiography. *Brain Res.* **655**: 237-240.

SZALLASI, A., NILSSON, S., FARKAS-SZALLASI, T., BLUMBERG, P.M., HÖKFELT, T. AND LUNDBERG, J.M., (1995) Vanilloid (capsaicin) receptors in the rat: distribution in the brain, regional differences in the spinal cord, axonal transport to the periphery, and depletion by systemic vanilloid pretreatment. *Brain Res.* **703**: 175-183.

SZALLASI, A., ACS, G., CRAVOTTO, G., BLUMBERG, P.M., LUNDBERG, J.M., APPENDINO, G. (1996) A novel agonist, phorbol 12-phenylacetate 13-acetate 20-homovanillate, abolishes positive cooperativity of binding by the vanilloid receptor. *Eur. J. Pharmacol.* **299**: 221-228.

SZOLCSÁNYI, J. (1993) Actions of capsaicin on sensory receptors. In: *Capsaicin in the Study of Pain*. J.N. Wood (ed.), pp. 1-27, Academic Press Ltd., London.

SZOLCSÁNYI, J. AND JANSCÓ-GÁBOR, A. (1975) Sensory effects of capsaicin congeners I: relationship between chemical structure and pain-producing potency of pungent agents. *Drug Res.* **25**: 1877-1881.

SZOLCSÁNYI, J. AND JANSCÓ-GÁBOR, A. (1976) Sensory effects of capsaicin congeners II: importance of chemical structure and pungency in desensitising activity of capsaicin-type compounds. *Drug Res.* **26**: 33-37.

TONG, A.C.Y., RATTIGAN, S. AND CLARK, M.G. (1998) Similarities between vasoconstrictor- and veratridine-stimulated metabolism in perfused rat hindlimb. *Can. J. Physiol. Pharmacol.* In Press.

URBAN, L. AND DRAY, A. (1991) Capsazepine, a novel capsaicin antagonist, selectively antagonises the effects of capsaicin in the mouse spinal cord *in vitro*. *Neurosci. Lett.* **134**: 9-11.

VASINGTON, F.D., GAZZOTTI, P., TIOZZO, R. AND CARAFOLI, E. (1972) The effect of ruthenium red on Ca^{2+} transport and respiration in rat liver mitochondria. *Biochim. Biophys. Acta* **256**: 43-54.

VIGNA, S.R., BOWDEN, J.J., MCDONALD, D.M., FISHER, J., OKAMOTO, A., MCVEY, D.C., PAYAN, D.G. AND BUNNETT, N.W. (1994) Characterisation of antibodies to the rat substance P (NK1) receptor and to a chimeric substance P receptor expressed in mammalian cells. *J. Neurosci.* **14**: 834-845.

WALLACE, W.F.M. (1997) Contribution of nitric oxide to vasodilatation by substance P in human forearm vessels. *Clin. Sci.* **92**: 607.

WALPOLE, C.S.J. AND WRIGGLESWORTH, R. (1993) Structural requirements for capsaicin agonists and antagonists. In: *Capsaicin in the Study of Pain*. J.N. Wood (ed.), pp. 63-82, Academic Press Ltd., London.

WALPOLE, C.S.J., WRIGGLESWORTH, R., BEVAN, S., CAMPBELL, E.A., DRAY, A., JAMES, I.F., PERKINS, M.N., REID, D.J. AND WINTER, J. (1993a) Analogues of capsaicin with agonist activity as novel analgesic agents; structure-activity studies. 1. The aromatic "A-region". *J. Med. Chem.* **36**: 2362-2372.

WALPOLE, C.S.J., WRIGGLESWORTH, R., BEVAN, S., CAMPBELL, E.A., DRAY, A., JAMES, I.F., MASDIN, K.J., PERKINS, M.N., REID, D.J. AND WINTER, J. (1993b) Analogues of capsaicin with agonist activity as novel analgesic agents; structure-activity studies. 1. The amide bond "B-region". *J. Med. Chem.* **36**: 2373-2380.

WALPOLE, C.S.J., WRIGGLESWORTH, R., BEVAN, S., CAMPBELL, E.A., DRAY, A., JAMES, I.F., MASDIN, K.J., PERKINS, M.N., REID, D.J. AND WINTER, J. (1993c) Analogues of capsaicin with agonist activity as novel analgesic agents; structure-activity studies. 1. The hydrophobic side-chain "C-region". *J. Med. Chem.* **36**: 2381-2389.

WALPOLE, C.S.J., BEVAN, S., BOVERMANN, G., BOELSTERLI, J.J., BREDKENDRIDGE, R., DAVIES, J.W., HUGHES, G.A., JAMES, I.F AND OBERER, L. (1994) Discovery of capsazepine: the first competitive antagonist of the sensory neuron excitants capsaicin and resiniferatoxin. *J. Med. Chem.* **37**: 1942-1954.

WALPOLE, C.S.J., BEVAN, S., BLOOMFIELD, G., BREDKENDRIDGE, R., JAMES, I.F., RITCHIE, T., SZALLASI, A., WINTER, J., AND WRIGGLESWORTH, R. (1996) Similarities and differences in the structure-activity relationships of capsaicin and resiniferatoxin analogues. *J. Med. Chem.* **39**: 2939-2952.

WANG, Z.Y. AND HÅKANSON, R. (1993) Effects of ruthenium red and capsazepine on C-fibres in the rabbit iris. *Br. J. Pharmacol.* **110**: 1073-1078.

WANG, Z.Y., TUNG, S.R., STRICHARTZ, G.R. AND HÅKANSON, R. (1994) Non-specific actions of the non-peptide tachykinin antagonists CP 96,345, RP 67,580 and SR 48968, on neurotransmission. *Br. J. Pharmacol.* **111**:179-184.

WARDLE, K.A., FUREY, G. AND SANGER, G.J. (1996) Pharmacological characterization of the vanilloid receptor in the rat isolated *vas deferens*. *J. Pharm. Pharmacol.* **48**: 285-291,

WATANABE, T., KAWADA, T. AND IWAI, K. (1987) Enhancement by capsaicin of energy metabolism in rats through secretion of catecholamine from adrenal medulla. *Agric. Biol. Chem.* **51**: 75-79.

WATANABE, T., KAWADA, T., KUROSAWA, M., SATO, A. AND IWAI, K. (1991) Thermogenic action of capsaicin and its analogues. In: *Obesity: Dietary Factors and Control.* D.R. Romos *et al.* (eds.), pp. 67-77, Japan Science Society Press.

WATLING, K.J. (1992) Non-peptide antagonists herald a new era in tachykinin research. *Trends Pharmacol. Sci.* **13**: 266-269.

WATLING, K.J. AND KRAUSE, J.E. (1993) The rising sun shines on substance P and related peptides. *Trends Pharmacol. Sci.* **14**: 81-84.

WEBB-PEPLOE, M.M., BRENDER, D. AND SHEPHERD, J.T. (1972) Vascular responses to stimulation of receptors in muscle by capsaicin. *Am. J. Physiol.* **222**: 189-195.

WHITE, C.B., ROBERTS, A.M. AND JOSHUA, I.G. (1993) Arteriolar dilation mediated by capsaicin and calcitonin gene-related peptide in rats. *Am. J. Physiol.* **265** (*Heart Circ. Physiol.* 34): H1411-1415.

WHITTLE, B.J.R., LOPEZ-BELMONTE, J. AND REES, D.D. (1989) Modulation of the vasodepressor actions of acetylcholine, bradykinin, substance P and endothelin in the rat by a specific inhibitor of nitric oxide formation. *Br. J. Pharmacol.* **98**: 646-652.

WINTER, J., DRAY, A., WOOD, J.N., YEATS, J.C. AND BEVAN, S. (1990) Cellular mechanisms of action of resiniferatoxin: a potent sensory neuron excitotoxin. *Brain Res.* **520**: 131-140.

WOOD, J.N., (Ed.) (1993) *Capsaicin in the study of pain*, Academic Press, New York.

WOOD, J.N., WINTER, J., JAMES, I.F., RANG, H.P., YEATS, J., AND BEVAN, S. (1988) Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J. Neurosci.* **8**: 3208-3220.

WRIGGLESWORTH, R. WALPOLE , C.S.J., BEVAN, S., CAMPBELL E.A., DRAY A., HUGHES G.A., JAMES I., MASDIN K.J. AND WINTER J. (1996) Analogues of capsaicin with agonist activity as novel analgesic agents - structure-activity studies for potent, orally active analgesics. *J. Med. Chem.* **39**: 4942-4951.

YAMADA, M., ISHIKAWA, T., FUJIMORI, A., AND GOTO, K. (1997a) Local neurogenic regulation of rat hindlimb circulation - role of calcitonin gene-related peptide in vasodilatation after skeletal muscle contraction. *Br. J. Pharmacol.* **122**: 703-709.

YAMADA, M., ISHIKAWA, T., YAMANAKA, A., FUJIMORI, A., AND GOTO, K. (1997b) Local neurogenic regulation of rat hindlimb circulation - CO_2-induced release of calcitonin gene-related peptide from sensory nerves. *Br. J. Pharmacol.* **122**: 710-714.

YAMANAKA, K., KIGOSHI, S. AND MURAMATSU, I. (1984) Conduction block induced by capsaicin in crayfish giant axon. *Brain Res.* **300**: 113-119.

YE, J-M., COLQUHOUN, E.Q., HETTIARACHCHI, M., AND CLARK, M.G. (1990) Flow-induced oxygen uptake by the perfused rat hindlimb is inhibited by vasodilators and augmented by norepinephrine: a possible role for the microvasculature in hindlimb thermogenesis. *Can. J. Physiol. Pharmacol.* **68**: 119-125.

YE, J-M., CLARK, M.G. AND COLQUHOUN, E.Q. (1995) Constant pressure perfusion of rat hindlimb shows α- and β-adrenergic stimulation of oxygen consumption. *Am. J. Physiol.* **269**: E960-E968.

YOKOTA, Y., SASAI, Y., TANAKA, K., FUJIWARA, T., TSUCHIDA, K., SHIGEMOTO, R., KAKIZUKA, A., OHKUBO, H. AND NAKANISHI, S. (1989) Molecular characterisation of a functional cDNA for rat substance P receptor. *J. Biol. Chem.* **264**: 17649-17652.

ELSEVIER

Life Sciences, Vol. 59, No. 2, pp. 105-117, 1996

0024-3205/96 $15.00 + .00

PII S0024-3205(96)00267-6

CAPSAICIN-INDUCED BIPHASIC OXYGEN UPTAKE IN RAT MUSCLE: ANTAGONISM BY CAPSAZEPINE AND RUTHENIUM RED PROVIDES FURTHER EVIDENCE FOR PERIPHERAL VANILLOID RECEPTOR SUBTYPES (VN_1/VN_2)

Cory D. Griffiths, Tristram P. D. Eldershaw, *Dominic P. Geraghty, Jennifer L. Hall and Eric Q. Colquhoun

Division of Biochemistry, Faculty of Medicine and Pharmacy, University of Tasmania, Hobart, Australia 7001, and *Department of Biomedical Science, University of Tasmania, Launceston, Australia 7250

(Received in final form May 6, 1996)

Summary

Previous studies with the vanilloid spice principle capsaicin have demonstrated a biphasic VO_2 response, with vasoconstriction, in the perfused rat hindlimb that has led to suggestions of vanilloid receptor subtypes (VN_1/VN_2) in this preparation (1). In the present study, the known competitive vanilloid antagonist capsazepine inhibited the above capsaicin-mediated effects in a manner that was indicative of binding at specific vanilloid recognition sites. Low concentrations of capsazepine selectively inhibited the increased VO_2 produced by the putative VN_1 receptor at submicromolar concentrations of capsaicin, while the inhibition of VO_2 produced by high concentrations of capsaicin (putative VN_2) was enhanced. These observations, showing different susceptibilities to blockade by capsazepine, further support the presence of two vanilloid receptor subtypes in the rat hindlimb. Schild plots of the data yielded variable slopes that approach unity at greater responses to capsaicin (mean K_B = 8.44 ± 2.08 μM and 7.28 ± 0.78 μM for VO_2 and perfusion pressure curves, respectively). Low concentrations of the capsaicin antagonist ruthenium red selectively blocked the putative VN_2 receptor-mediated effects produced by high concentrations of capsaicin. The non-competitive nature of this inhibitor suggests an operation through separate receptor-coupled ion channel complexes at high and low concentrations of the vanilloid. Tetrodotoxin failed to attenuate any changes produced by capsaicin, suggesting that the mechanism of action of capsaicin in the rat hindlimb may differ from other tissues.

Key Words: capsaicin, capsazepine, ruthenium red, receptor subtypes, oxygen consumption, vasoconstriction

The mechanism by which vanilloid spice principles elicit their effects on a variety of target tissues has received particular attention in recent reviews (2, 3). Most studies on the actions of the vanilloids have been restricted largely to capsaicin, from the fruit of *Capsicum sp.*, a compound best

Correspondence to Eric Q. Colquhoun

known for its excitatory, desensitising and toxic effects on a subset of unmyelinated (C-) or thinly myelinated (Aδ-) sensory neurones (4). The sensitivity of these neurones to capsaicin is likely to be due to the presence of a cation channel that, when stimulated by vanilloids, allows the influx of various cations facilitating the release of several neuropeptides (2, 5).

Substantial evidence has now accumulated indicating that the actions of both capsaicin and the ultrapotent analogue resiniferatoxin (RTX) are mediated via a specific vanilloid recognition site that is believed to be related intimately with the capsaicin-operated cation channel (6). This notion has been strengthened by the recent development of capsazepine, the first competitive antagonist shown to be selective for the actions of capsaicin on central nerve endings in the rat (7, 8), in functional studies on capsaicin-mediated ion uptake in the same neurones (9), and contraction of smooth muscle (10).

Radioligand binding studies using [^{3}H]-RTX have permitted the biochemical characterisation of the vanilloid receptor (11-13) and have demonstrated species heterogeneity, as well as possible intraspecies receptor subtypes (14). Peripheral vanilloid receptors in the urinary bladder (15), colon (16), and urethra (17) of the rat all bind [^{3}H]-RTX in a non-cooperative fashion, and show decreased affinity for RTX, that is distinct from the positive cooperativity of [^{3}H]-RTX binding central receptors (13, 15). Additionally, capsaicin inhibits specific [^{3}H]-RTX binding to central vanilloid receptors with a 7 to 10-fold higher affinity than the competitive antagonist capsazepine (18), while at peripheral receptors in the colon, the order of potency is reversed (16).

The evidence for peripheral vanilloid receptor subtypes is limited to RTX showing some differential affinity for receptor binding in the colon (16). On the other hand, Liu and Simon (19) have provided evidence for multiple central nervous system vanilloid receptor types where patch-clamped rat trigeminal neurones exhibit two types of inward current (one fast and one slow activating/inactivating) in response to capsaicin, both of which are sensitive to capsazepine and ruthenium red.

In our laboratory, capsaicin, and a range of other vasoconstricting vanilloids, have been shown to stimulate oxygen consumption (VO_2) in the perfused iliac bed of the rat at high nanomolar concentrations, while micromolar concentrations of vanilloid reduce VO_2 to levels well below basal values (20-22). Both effects are associated with concentration-dependent increases in perfusion pressure, indicating constriction of the hindlimb vasculature. The biphasic action on VO_2 in the hindlimb is not solely a concentration-dependent feature of capsaicin and other vanilloids. Studies from our laboratory (23) and similar work by others (24, 25) have confirmed a similar biphasic response to noradrenaline, but with stronger responses at comparatively lower concentrations than those observed with the vanilloids. While no definitive link between VO_2 and perfusion pressure responses has been established, two possible explanations from our studies in the hindlimb centre on either working (contracting) vascular smooth muscle as oxygen consuming tissue, or site-specific receptors promoting flow redistribution within skeletal muscle (reviewed in 26).

Recently, we have postulated the presence of two different vanilloid receptor types (designated VN_1 and VN_2) in rat hindlimb tissue based on both functional and metabolic evidence (1). Putative VN_1 and VN_2 receptors were distinguished on the basis of their differing affinities for capsaicin, their different calcium and oxygen dependencies for inducing vasoconstriction, and their ability to stimulate or inhibit VO_2. In the present communication, we provide further evidence for the presence of dual vanilloid receptors in the same preparation. This evidence is the result of studying the effects of the selective competitive vanilloid antagonist capsazepine, and the selective non-

competitive antagonist ruthenium red on capsaicin-mediated changes in VO_2 and perfusion pressure. Although non-competitive, ruthenium red is thought to selectively impair the vanilloid receptor-channel coupling mechanism by a rapid blocking and unblocking of the stimulated cation channel (27). These antagonists have proved to be valuable in the present study in determining the nature of the biphasic VO_2 responses produced by vanilloids in the perfused rat hindlimb.

Additionally, the neurotoxin tetrodotoxin (TTX) was employed to assess the possibility that capsaicin acts through specific sensory neurones where dual cellular mechanisms are believed to operate (28). Maggi (29) has summarised the available evidence indicating the presence of these mechanisms for the release of sensory transmitters from capsaicin-sensitive primary afferent neurones, one being TTX-sensitive (low capsaicin concentrations) while the other is TTX-resistant (high capsaicin concentrations). In the present study, we have also co-infused tetrodotoxin and capsaicin in an attempt to distinguish between these modes of action if they are present in rat muscle.

Methods

Rat hindlimb perfusion

All experimental procedures used were approved by the University of Tasmania Animal Ethics Committee under the Australian code of practice for the care and use of animals for scientific purposes (30).

Perfusions were conducted using male, 180 to 200g hooded Wistar rats fed a commercial rat chow diet containing 21.4% protein, 4.6% lipid, 68% carbohydrate and 6% crude fibre with added vitamins and minerals (Gibson's, Hobart). Animals were housed at 21±1°C under a 12h:12h light/dark cycle in groups with an accessible and plentiful water supply.

Anaesthesia, surgery and perfusion procedures were performed as described previously (31). Briefly, the left hindlimb of the rat was perfused at 25°C with a modified Krebs-Ringer bicarbonate buffer containing 8.3mM glucose, 1.27mM $CaCl_2$ and 2% w/v dialyzed bovine serum albumin (Fraction V). The perfusate reservoir was gassed with 95% O_2-5% CO_2 at 4°C and pumped by a peristaltic pump at a constant rate of 4.0±0.1 ml/min through a heat exchange coil, maintained at 25°C, and a sealed silastic lung continually gassed with the above O_2/CO_2 mixture. This ensured constant perfusate temperature and arterial PO_2 levels. The medium entered and exited the rat hindlimb via cannulas inserted into the aorta (Surflo I.V. catheter, 20G, Terumo, USA) and vena cava (Surflo I.V. catheter, 16G, Terumo, USA) respectively.

In perfusions involving sympathetic nerve stimulation the lumbar sympathetic trunk was ligated, cut and pulled perpendicular to the aorta in contact with two stainless steel electrodes connected to a stimulator. Liquid paraffin and parrafin film were placed under the electrodes to prevent stimulation of surrounding tissues. The hindlimb was perfused with the above medium containing 1 µM tubocurarine to prevent motor neurone activity. After equilibration, the sympathetic trunk was stimulated with 5V square waves of 5 ms pulse width with variable frequency.

The oxygen content of the venous effluent was measured continuously using an in-line 0.5ml Clark-type oxygen electrode while perfusion (arterial) pressure was monitored by means of a pressure transducer proximal to the aorta. The method of calculation of VO_2 has been described previously (31). Values for VO_2 calculation and perfusion pressure were taken only after steady state conditions were obtained.

Agent infusion

The infusion of various agents into the hindlimb occurred only after the perfusion had reached steady state VO_2 and pressure. All agents were either freshly prepared before each experiment, or prepared and then stored at 4°C if chemically stable. Due to the lipophilic nature of vanilloids and their affinity for silicon-based tubing, capsaicin was dissolved in 50% ethanol and infused using a syringe pump (Model 2620, Harvard apparatus, USA) driving a 1.0 ml glass syringe equipped with teflon tubing. All other agents were infused with a second pump (Model 355, Sage instruments, Orion Research Inc., USA) using an identical 1.0 ml glass syringe. Capsazepine was dissolved in 70-80% ethanol, ruthenium red and tetrodotoxin in glass-distilled water, the latter with 5 mg citrate buffer contained in a sealed vial. Where ethanol was used as the vehicle, special care was taken to limit the infusion rate (usually to below 10 µl/min) to avoid the perturbation of effects by this organic solvent.

For all experiments, the concentration-response curves for capsaicin were constructed in a cumulative manner with at least two low (submicromolar) doses and two high (micromolar) doses. The addition of each dose occurred only after VO_2 and perfusion pressure steady state was attained with the preceding dose. Where the effects of capsaicin were examined against capsazepine, ruthenium red, or tetrodotoxin, the relevant inhibitor was first infused alone to detect any changes that these drugs may elicit. Increasing concentrations of capsaicin were subsequently co-infused. After the removal of the final capsaicin concentration, the inhibitor was also removed and the hindlimb was allowed to return to basal values of steady state VO_2 and perfusion pressure.

In a separate set of control perfusions, capsazepine and ruthenium red were tested against the VO_2 and perfusion pressure changes induced by serotonin (5-HT) (0.35 µM), low (50 nM) and high (5 µM) concentrations of noreadrenaline, and angiotensin II (3 nM) to establish selectivity of the antagonists for the effects of capsaicin. Tetrodotoxin (0.3 µM) was infused against the VO_2 and perfusion pressure changes induced by low (0.5 Hz) and high (5 Hz) frequency sympathetic nerve stimulation.

Statistical analysis

Statistical analysis of the data was performed by use of Student's *t*-test or by analysis of variance (ANOVA), where applicable. All values are given as the mean ± standard error (s.e.) mean. The Schild plots were constructed from VO_2 and perfusion pressure log concentration-response curves. Dose ratios were estimated at responses between 15% and 85% of maximum.

Drugs and chemicals

Bovine serum albumin (Fraction V) was purchased from Boehringer Mannheim (Australia); capsaicin, noradrenaline, angiotensin II, serotonin, ruthenium red, and tetrodotoxin from Sigma (USA); capsazepine from Research Biochemicals International (USA); pentobarbitone sodium (Nembutal, 60 mg/ml) from Bomac Laboratories Pty. Ltd. (Australia); heparin sodium from David Bull Laboratories (Australia); NaCl, KCl, KH_2PO_4, $MgSO_4$, $NaHCO_3$, $CaCl_2$ and D-Glucose from Ajax Chemicals Ltd. (Australia).

Results

For experiments with capsazepine, the mean basal arterial PO_2 was 695.7±5.1 mm Hg and the unstimulated mean venous PO_2 was 366.9±10.3 mm Hg (n=31). Mean basal oxygen consumption (VO_2) and perfusion pressure were 7.9±0.3 µmol. $g.^{-1}$ h^{-1} and 28.5±0.7 mm Hg respectively (n=31).

Capsaicin produced a concentration-dependent biphasic response in VO_2 (Fig. 1A) and an associated vasoconstriction-induced rise in perfusion pressure (Fig. 1B) matching that seen by Colquhoun *et al.* (1). A maximum increase in VO_2 (1.2±0.1 μmol. $g.^{-1}$ h^{-1}) was observed at 0.5 μM capsaicin coupled with an increase in perfusion pressure of 9.0±0.9 mm Hg. The highest concentration of capsaicin used (5 μM) produced a strong inhibition of VO_2 (1.9±0.2 μmol. $g.^{-1}$ h^{-1} below basal) with a greater increase in perfusion pressure of 31.6±2.1 mm Hg.

The infusion of capsazepine alone at the concentrations used to inhibit responses to capsaicin failed to alter any basal parameters, and is therefore unlikely to be intrinsically active. Capsazepine did not cause statistically significant alterations ($P \geq 0.05$) in VO_2 and perfusion pressure changes induced by serotonin (5-HT), angiotensin II, or low and high concentrations of noradrenaline (data not shown).

Capsazepine (0.32-32 μM) produced a concentration-dependent rightward shift in the concentration response curves to capsaicin, most evident in the perfusion pressure changes (Fig. 1B) and inhibition of VO_2 (Fig. 1A). The slopes of Schild plots constructed from dose ratios estimated at various levels of VO_2 inhibition show that as the response becomes greater (i.e. greater inhibition of VO_2) the Schild regressions approach a slope close to unity (Fig. 2A.).

With the infusion of 3.2 μM and 10 μM capsazepine, a maximal perfusion pressure response was achieved with the addition of 5 μM of the agonist (capsaicin). This maximal concentration increased to 20 μM capsaicin at the highest dose of capsazepine (32 μM). Schild regressions constructed from the perfusion pressure data (Fig. 2B.) showed a similar pattern to those for VO_2 in that a decrease in slope is evident from dose ratios estimated at greater responses to capsaicin, although they differ in the range of their slopes and the points of intercept on the abscissa. Thus for Fig. 2A the slopes decreased progressively from 1.81 to 1.01, whereas the slopes from Fig. 2B decreased from 2.04 to 1.45.

In contrast to Fig. 1B, Fig. 1A shows aberrant behavior with capsaicin in the presence of capsazepine. At a low concentration of capsaicin (0.5 μM), low concentrations of capsazepine cause an initial fall in response (decreased stimulation of VO_2), followed by a temporary increase in response at higher concentrations of the antagonist (see (i) inset Fig. 1A). A further increase in capsazepine concentration caused a second but steady decrease in response. At higher concentrations of capsaicin (2-5 μM), the VO_2 response is initially enhanced by low concentrations of capsazepine (enhanced inhibition of VO_2) followed by a steady decrease in response at higher concentrations of the antagonist (see (ii) inset Fig. 1A). It appears, therefore, that low concentrations of capsazepine are selectively blocking the stimulatory VO_2 effects produced by low concentrations of capsaicin, resulting in the enhancement of the inhibitory VO_2 effects produced by high concentrations of the vanilloid.

Near maximal inhibition of VO_2 was obtained at high concentrations of the agonist with all concentrations of capsazepine. Maximal low dose responses (stimulatory on VO_2) were not achieved at capsazepine concentrations of 1, 10 or 32 μM, an observation likely to be a function of the capsaicin concentrations chosen in the initial infusion protocol.

Infusion of ruthenium red (0.01-1.0 μM) alone did not affect mean basal arterial PO_2 (702.6±7.9 mm Hg), venous PO_2 (419.3±4.6 mm Hg), VO_2 (6.8±0.3 μmol. $g.^{-1}$ $h.^{-1}$), or perfusion pressure (24.8±0.3 mm Hg, n=4). The antagonist also failed to show statistically significant inhibition ($P \geq 0.05$) of the VO_2 and perfusion pressure changes induced by serotonin, angiotensin II, and low and high concentrations of noradrenaline (data not shown).

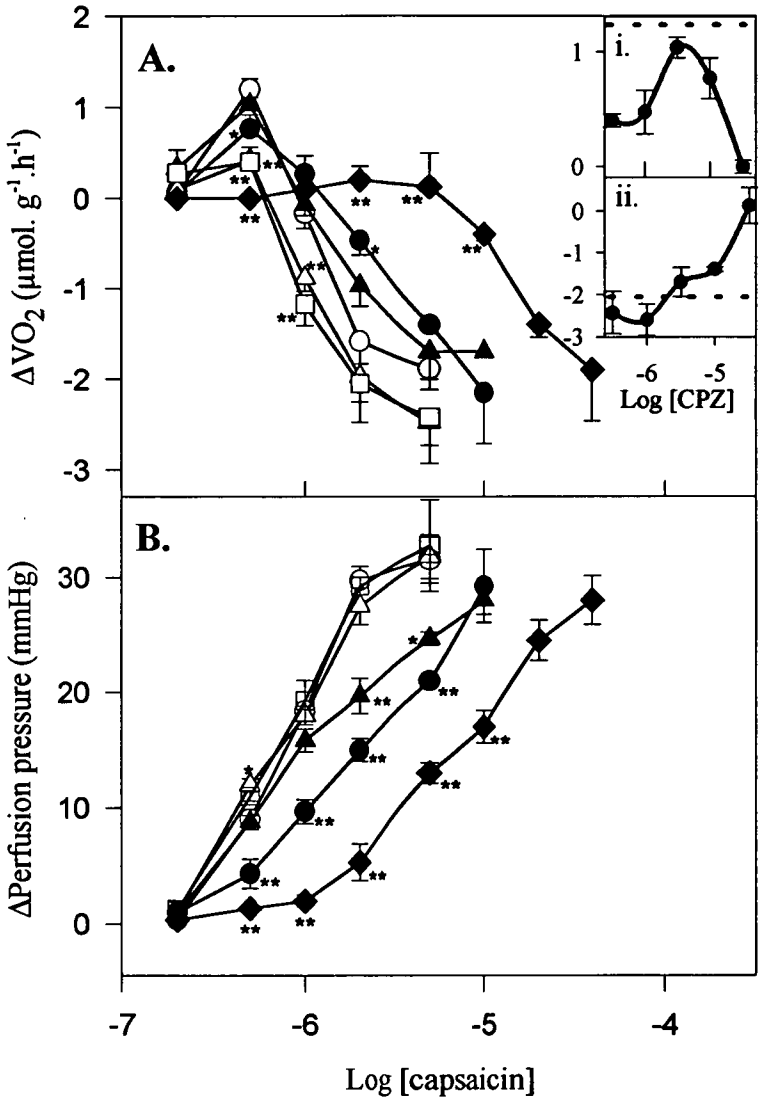


Fig. 1

Effect of capsazepine (CPZ) on concentration-response curves for capsaicin-induced changes in **A.** oxygen consumption (VO_2), and **B.** perfusion pressure in the isolated perfused rat hindlimb: control (○); 0.32 μM CPZ (□); 1.0 μM CPZ (Δ); 3.2 μM CPZ (▲); 10.0 μM CPZ (●); and 32.0 μM CPZ (◆) in 4-11 experiments. All values are mean ± s.e. mean, *P≤0.05 **P≤0.01 (ANOVA) against control. Inset (panel A) shows the ΔVO_2 response to (i) 5 x 10^{-7} M and (ii) 5 x 10^{-6} M capsaicin with increasing concentrations of CPZ. The dotted line shows the control ΔVO_2 at these concentrations of capsaicin.

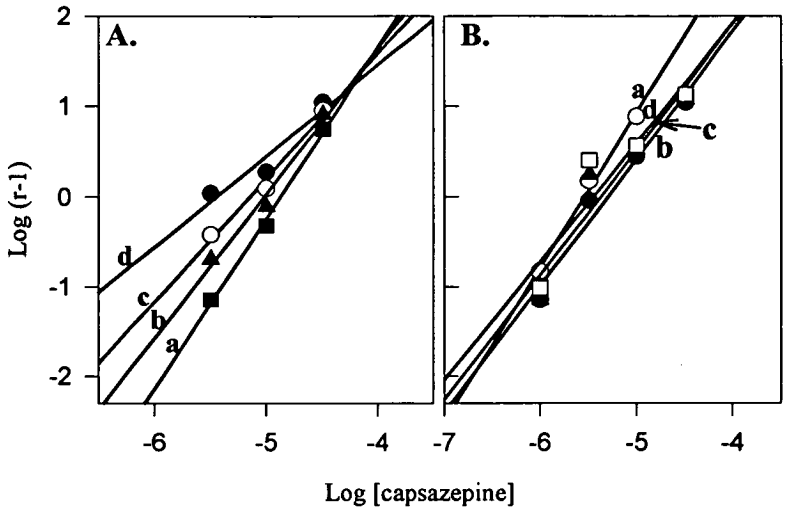


Fig. 2

Multiple Schild plots of the data presented in Fig. 1. **A.** Regressions were constructed from dose ratios estimated at four different levels of the inhibitory VO_2 response: **a.** 0 μmol. g^{-1}. h^{-1}, slope = 1.87; **b.** -0.5 μmol. g^{-1}. h^{-1}, slope = 1.61; **c.** -1.0 μmol. g^{-1}. h^{-1}, slope = 1.37; and **d.** -1.5 μmol. g^{-1}. h^{-1}, slope = 1.01. The mean K_B derived from the four Schild plots was 8.44 ± 2.08 μM. **B.** Regressions constructed from dose ratios estimated at four perfusion pressure responses: **a.** 15 mm Hg, slope = 2.04; **b.** 20 mm Hg, slope = 1.52, **c.** 23 mm Hg, slope = 1.49; and **d.** 25 mm Hg, slope = 1.45. Mean K_B from the four regressions was 7.28 ± 0.78 μM. Reduction in the number of plot points occurs where some concentration-response curves were shifted to the left of the control curves.

Ruthenium red produced a concentration-dependent inhibition of both VO_2 (Fig. 3A) and perfusion pressure (Fig. 3B) responses induced by capsaicin. For both VO_2 and perfusion pressure, the maximal response to the vanilloid agonist was markedly depressed in comparison to the control curve, and was almost completely abolished by the infusion of 1 μM ruthenium red. The stimulation of VO_2 at 0.5 μM capsaicin was inhibited by only 18% with the infusion of 0.1 μM ruthenium red. The same concentration of the dye attenuated the inhibition of VO_2 produced by both 2 μM and 5 μM capsaicin by 87% and 88% respectively.

A similar response was evident for perfusion pressure changes to the infusion of 0.5 μM and 5 μM capsaicin in the presence of 0.1 μM ruthenium red. The increase in pressure produced by the lower dose of capsaicin was inhibited by 32%, while that for the higher doses was reduced by 82%.

Tetrodotoxin (0.3μM) failed to inhibit VO_2 and perfusion pressure changes induced by low or high concentrations of capsaicin, but was effective at blocking similar changes induced by low and high frequency sympathetic nerve stimulation in the rat hindlimb preparation (data not shown).

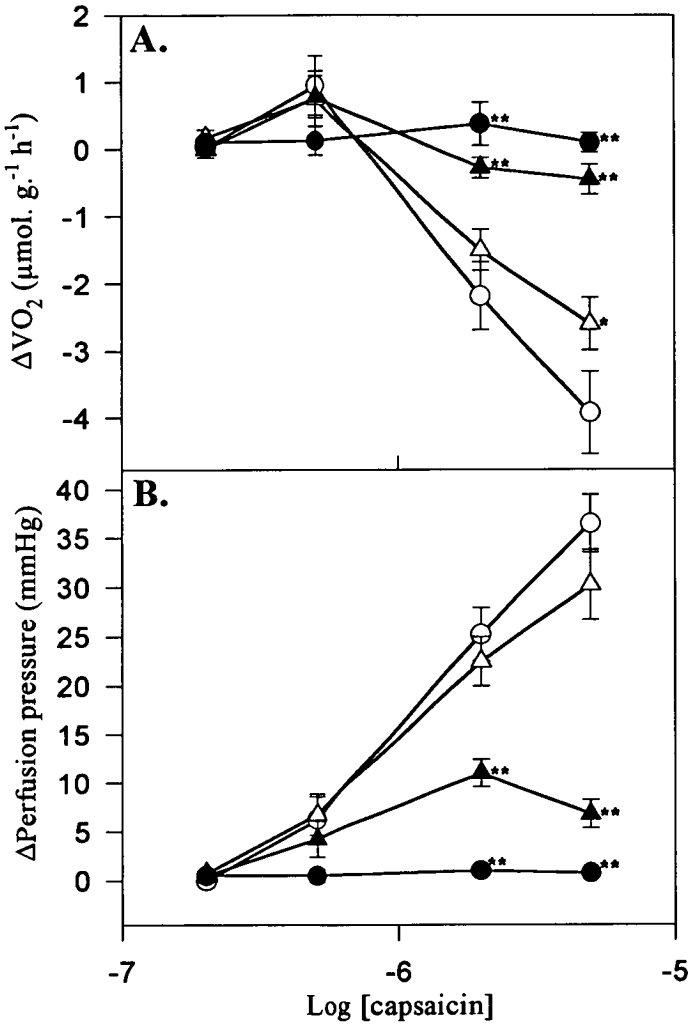


Fig 3

Effect of ruthenium red on concentration-response curves for capsaicin-induced changes in **A.** Oxygen consumption (VO_2); and **B.** perfusion pressure in the isolated perfused rat hindlimb: control (O); 0.01 µM ruthenium red (RR) (Δ); 0.1 µM RR (▲); and 1.0 µM RR (●) (all n=4). Values are mean ± s.e. mean. *P≤0.05 **P≤0.01 (ANOVA) against control.

Discussion

Capsaicin-induced vasoconstriction in isolated arteries has been attributed in the past to non-selective effects that occur via the direct physicochemical interaction of capsaicin with smooth

muscle plasma membranes, and do not exhibit desensitisation (reviewed by 2, 32). There are few reports, however, describing the effects of capsaicin on oxygen consumption in individual tissues. We believe that our previous (1, 20-22) and current findings represent a novel insight into the effects of capsaicin in peripheral tissues. While repeated application of vanilloids in the rat hindlimb did not produce desensitisation (22), a hallmark of the specific capsaicin action on sensory neurones, the present findings clearly indicate that the effects produced by capsaicin in this preparation are highly selective and very likely to be receptor-mediated.

Infusion of capsazepine into the rat hindlimb produced competitive antagonism of capsaicin-stimulated changes in VO_2 and perfusion pressure (Figs. 1A and 1B). The inability of capsazepine to inhibit other non-vanilloid agonists, that produce effects similar to those of capsaicin, suggests that capsazepine is highly specific for the actions of the vanilloid. Similar patterns of specific competitive antagonism by capsazepine have been observed in other systems where the effects of capsaicin are thought to be receptor-mediated (9, 10). Thus it is likely that capsaicin also operates via distinct vanilloid recognition sites in rat hindlimb tissue.

The reproducible biphasic nature of the capsaicin-induced VO_2 responses suggests the operation of a dual vanilloid receptor mechanism in the hindlimb preparation (1). These consistent observations are unlikely to be an artefact of these experiments as the perfusion technique used in these studies has been validated extensively in the past (see 26 for review).

The concept of one agonist acting on two opposing receptor types has been recently reviewed by Rovati and Nicosia (33) who attempted to distinguish the bell-shaped response curves produced by such interactions from those produced by partial agonists. A similar model developed earlier by Szabadi (34) proposed that an antagonist acting preferentially on one of the opposing receptor types will potentiate the effect produced by the other, and the net effect will be evident in the overall dose-response curve.

In the present investigation, we have been able to demonstrate similar effects by selectively inhibiting the stimulatory (low dose/VN_1) component of the biphasic VO_2 response to capsaicin with 0.32 μM and 1 μM capsazepine. This resulted in potentiation of the inhibitory (high dose/VN_2) VO_2 component. That is a single agonist (capsaicin) seems to act on two receptor types, one stimulatory and one inhibitory, to produce an overall biphasic curve, as demonstrated in Fig. 4. The VN_1 receptor is suggested to be responsible for the increases in VO_2 seen at submicromolar concentrations of capsaicin. The lower affinity VN_2 receptor produces a more pronounced inhibitory effect on VO_2 at capsaicin concentrations exceeding 1 μM. The theoretical curve VN_1+VN_2 represents the combined effect of the two receptors and models the biphasic curve derived from the infusion of increasing concentrations of capsaicin. We propose that low concentrations of capsazepine (0.32 μM and 1 μM) have selectively inhibited the putative VN_1 receptor and, consequently, the stimulation of VO_2 that it produces. Hence a shift in the balance of agonist occupancy has occurred in favour of the putative VN_2 (inhibitory) receptor. As a result, the inhibitory VO_2 response has become more pronounced in the overall concentration-response curves produced in the presence of low concentrations of capsazepine.

The complex interaction of agonist and antagonist interacting with two opposing receptor types, having different affinities, may have altered the dose ratios sufficiently to account for the steepness of some of the Schild regression slopes. We believe that as the inhibition of VO_2 becomes greater (by increasing capsaicin concentration) the emphasis of binding is placed in favour of the putative VN_2 receptor. As a result, the Schild plots derived at higher inhibitory VO_2 responses resemble more closely a single receptor-agonist/antagonist interaction. Steepness in Schild plots can also

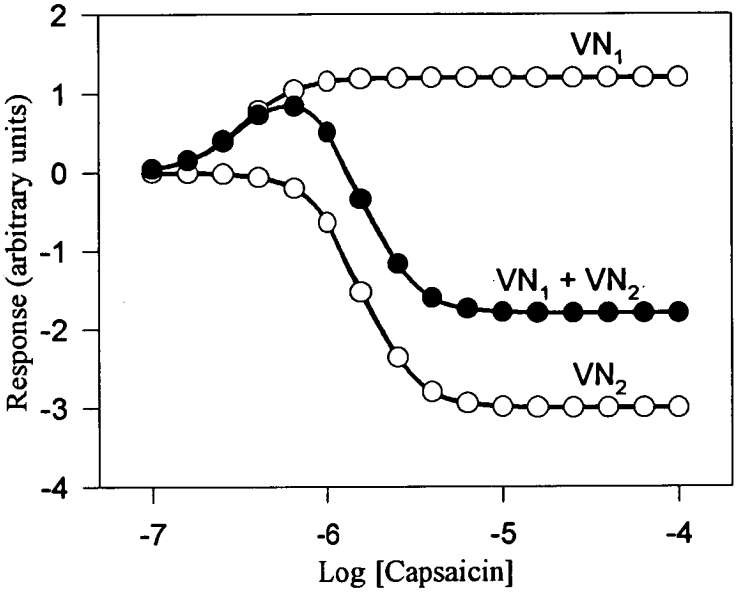


Fig. 4

A concentration-response curve model of oxygen consumption for putative vanilloid subtypes VN_1 and VN_2 in the perfused rat hindlimb. VN_1 and VN_2 curves were constructed by applying the four parameter logistic equation used by Rovati and Nicosia (33). EC_{50} (0.3 μM) and IC_{50} (1.6 μM) values for this equation were estimated from Fig.1A. Maximal VN_1 and VN_2 responses were estimated at 1.2 and -3.0 units, respectively. These receptor types show different affinities for capsaicin that is reflected in the observed concentration-response curve, represented by the sum of the theoretical VN_1 and VN_2 curves (VN_1+ VN_2). The inhibition of the VN_1 receptor type by low concentrations of capsazepine shifts the balance of agonist occupancy in favour of the opposing VN_2 receptor, hence causing the effect produced by this latter receptor type to predominate before it is also inhibited at higher capsazepine concentrations.

represent a non-equilibrium steady state between the antagonist and receptor, or the presence of a saturable antagonist removal mechanism (35). In this study, an antagonist removal mechanism may be present due to the use of bovine serum albumin (BSA) medium to act as the necessary oncotic agent. Recent trials in our laboratory have shown a 10-20 fold greater potency for capsaicin when 2% BSA is replaced by 4% Ficoll® (Pharmacia, Sweden) in the perfusion medium (data not shown), that is most likely due to the vanilloid binding to BSA. Capsazepine, being a structural analog of capsaicin, may also have a similar susceptibility to binding to BSA, however, on this basis it is difficult to account for the decreases in Schild plot slope when the dose ratios were estimated at greater agonist responses.

By contrast, Schild regressions obtained for the perfusion pressure data do not fully support our dual receptor proposal. These observations may be a result of the inability to distinguish between the similar vasoconstriction response produced by putative VN_1 and VN_2 receptors, unlike the

opposing responses on VO_2 where VN_1 and VN_2 effects can be distinguished with the infusion of low concentrations of capsazepine.

In the hindlimb preparation, 1 μM ruthenium red produced a specific but non-competitive inhibition of capsaicin-induced responses (Fig. 3) similar to that seen in the rat *vas deferens* and urinary bladder where the maximal responses to the vanilloid are potently antagonised (10). Again, this inhibition has been shown to be specific since the above concentration of ruthenium red used to block the effects of capsaicin did not alter the actions of other non-vanilloid agonists.

The infusion of submicromolar concentrations of the dye produced strong inhibition of the VO_2 and perfusion pressure effects of high (micromolar) concentrations of capsaicin, while low dose capsaicin responses showed only mild inhibition. We believe that the apparent selective antagonism of high dose capsaicin responses by low concentrations of ruthenium red further supports the notion of a dual vanilloid receptor system.

Given the suggested intimacy between putative vanilloid receptors and non-selective cation channels (6), it follows that if different vanilloid receptor types exist, then there could also be heterogeneity in the receptor-channel coupling system as a whole. The pattern of inhibition by low dose ruthenium red in rat hindlimb tissue certainly suggests that this may be the case and warrants further investigation.

Our observations with tetrodotoxin (TTX) in the perfused rat hindlimb are in contrast to those found by Lou *et al.* (28) in the perfused guinea-pig lung where the same concentration of the toxin (0.3 μM) inhibited the bronchoconstriction produced by low concentrations of capsaicin. The TTX-resistant nature of capsaicin-mediated changes in the rat hindlimb raises speculation regarding the cellular mechanisms promoted by vanilloids in this preparation. In view of recent reports by Akopian *et al.* (36) of a TTX-resistant voltage-gated sodium channel on capsaicin-sensitive rat dorsal root ganglion (DRG) neurones, it follows that capsaicin could possibly activate the opening of similar channels in rat skeletal muscle. However, attempts by these authors to detect such a channel in muscle have not been successful. Consequently, there exists the possibility that the cellular mechanisms promoted by capsaicin at low concentrations in the perfused rat hindlimb preparation could differ from other tissues, but more evidence is undoubtedly required.

At present, the role of sensory neuropeptides in capsaicin-mediated changes in the perfused hindlimb remains undetermined. Preliminary experiments in our laboratory with non-peptide neurokinin (NK, tachykinin) antagonists also suggest that only some of the actions mediated by the putative VN receptors are due to the release of tachykinins from sensory neurones (data not shown). Antagonists for other key neuropeptides are currently being examined including calcitonin gene-related peptide (CGRP) in light of recent suggestions for a role of this messenger in carbohydrate metabolism in skeletal muscle, and its release from sensory neurones in this tissue in response to vanilloids (37).

In conclusion, using specific competitive and non-competitive vanilloid receptor antagonists, we have obtained further evidence in support of the hypothesis of dual vanilloid receptors (VN_1 and VN_2 receptors) controlling the biphasic VO_2 responses to capsaicin in the perfused rat hindlimb (1). Although the perfusion pressure responses do not show a distinct dual receptor effect (eg. by a point of inflection in the perfusion pressure concentration-response curves) we believe that the inflection may be masked by an overlap in the VN_1 and VN_2 responses that produce the same positive effect on vascular tension.

Although the effects of capsaicin in the rat hindlimb are likely to be receptor and cation channel-mediated, the possibility exists that the post-receptor mechanisms are not entirely like those proposed for other systems given the resistance to TTX at all vanilloid concentrations tested. Capsaicin's action in the rat hindlimb may only partially involve the stimulation of sensory neurones, and release of neuropeptides, and may include direct interactions with vanilloid receptors in locations distinct from sensory neurones, such as vascular smooth muscle cells. However, the evidence supporting the above hypotheses is, at present, speculative and requires closer investigation.

Acknowledgments

This work has been supported in part by the National Health and Medical Research Council of Australia and the Australian Research Council.

References

1. E.Q. COLQUHOUN, T.P.D. ELDERSHAW, K.L. BENNETT, J.L. HALL, K.A. DORA, & M.G. CLARK, Life Sci. **57** 91-102 (1995).
2. P. HOLZER, Pharmacol. Rev. **42** 143-201 (1991).
3. S.J. BEVAN & R.J. DOCHERTY, *Capsaicin in the study of pain.* Wood, J.N. (Ed.), 27-44, Academic Press Ltd, London (1993).
4. J.N. WOOD, J. WINTER, I.F. JAMES, H.P. RANG, J. YEATS, & S. BEVAN, J. Neurosci. **8** 3208-3220 (1988).
5. S. BEVAN & J. SZOLSCANYI, Trends Pharmacol. Sci. **11** 330-333 (1991).
6. I.F. JAMES, N. NINKINA, & J.N. WOOD,. *Capsaicin in the study of pain,*. J.N. Wood, (Ed.), 27-44, Academic Press Ltd, London (1993).
7. L. URBAN & A. DRAY, Neurosci. Lett. **134** 9-11 (1991).
8. A.H. DICKENSON & A. DRAY, Br. J. Pharmacol. **104** 1045-1049 (1991).
9. S.J. BEVAN, S. HOTHI, G. HUGHES, I.F. JAMES, H.P. RANG, K. SHAH, C.S.J. WALPOLE, & J.C. YEATS, Br. J. Pharmacol. **107** 544-552 (1992).
10. C.A. MAGGI, S. BEVAN, C.S.J. WALPOLE, H.P. RANG, & S. GIULIANI, Br. J. Pharmacol. **108** 801-805 (1993).
11. A. SZALLASI & P.M. BLUMBERG, Brain Res. **524** 106-111 (1990a).
12. A. SZALLASI & P.M. BLUMBERG, Life Sci. **47** 1399-1408 (1990b).
13. A. SZALLASI & P.M. BLUMBERG, Naunyn-Schmiedeberg's Arch. Pharmacol. **347** 84-91 (1993).
14. A. SZALLASI, Gen Pharmac. **25** 223-243 (1994).
15. A. SZALLASI, B. CONTE, C. GOSO, P.M. BLUMBERG, & S. MANZINI, Life Sci. **52** PL221-226 (1993).
16. C. GOSO, S. EVANGELISTA, M. TRAMONTANA, S. MANZINI, P.M. BLUMBERG, & A. SZALLASI, Eur. J. Pharmacol. **249** 185-190 (1993).
17. M. PARLANI, B. CONTE, C. GOSO, A. SZALLASI, & S. MANZINI, Br. J. Pharmacol. **110** 989-994 (1993).
18. C. GOSO, S. MANZINI, P.M. BLUMBERG, & A. SZALLASI, Br. J. Pharmacol. **108** (Suppl.), 306P (1993).
19. L. LIU & S.A. SIMON, Proc. Natl. Acad. Sci. **91** 738-741 (1994).

20. D. CAMERON-SMITH, E.Q. COLQUHOUN, J-M YE, M. HETTIARACHCHI, & M.G. CLARK, Int. J. Obesity **4** 259-270 (1990).
21. T.P.D. ELDERSHAW, E.Q. COLQUHOUN, K.A. DORA, Z-C. PENG, & M.G. CLARK, Int. J. Obesity. **16** 755-763 (1992).
22. T.P.D. ELDERSHAW, E.Q. COLQUHOUN, K.L. BENNETT, K.A. DORA, & M.G. CLARK, Life Sci. **55** 389-397 (1994).
23. K.A. DORA, S.M. RICHARDS, S. RATTIGAN, E.Q. COLQUHOUN, & M.G. CLARK, Am. J. Physiol. **262** (Heart Circ. Physiol. 31), H698-H703 (1992).
24. B. GRUBB, & G.E. FOLK, J. Comp. Physiol. **110** 217-226 (1976).
25. C. CÔTÉ, M.C. THIBAULT, & J. VALLIÈRES, Life Sci. **37** 695-701 (1985).
26. M.G. CLARK, E.Q. COLQUHOUN, S. RATTIGAN, K.A. DORA, T.P.D. ELDERSHAW, J.L. HALL, & J-M. YE, Am. J. Physiol. **268** (Endocrinol. Metab. 31), E797-E812 (1995).
27. A. DRAY, C.A. FORBES, & G.M. BURGESS, Neurosci. Lett. **110** 52-59 (1990).
28. Y-P. LOU, A. FRANCO-CERECEDA, & J.M. LUNDBERG, Acta Physiol. Scand. **146** 119-127 (1992).
29. C.A. MAGGI, *Capsaicin in the study of pain.* J.N. Wood (Ed.), 161-190 Academic Press Ltd, London (1993).
30. AUSTRALIAN GOVERNMENT, *Australian code of practice for the care and use of animals for scientific purposes.* Australian Government Publishing Service, Canberra (1990).
31. E.Q. COLQUHOUN, M. HETTIARACHCHI, J-M. YE, E.A. RICHTER, A.J. HNIAT, S. RATTIGAN, & M.G. CLARK, Life Sci. **43** 1747-1754 (1988).
32. C.A. MAGGI & A. MELI, Gen. Pharmac. **19** 1-43 (1988)
33. G.E. ROVATI, & S. NICOSIA, Trends Pharmacol. Sci. **15** 140-144 (1994).
34. E. SZABADI, J. Theor. Biol. **69** 101-112 (1977).
35. T. KENAKIN, *Pharmacologic analysis of drug-receptor interaction* (2nd edition), Raven Press Ltd., New York (1993).
36. A.N. AKOPIAN, L. SIVILOTTI & J.N. WOOD, Nature **379** 257-262 (1996).
37. B. LEIGHTON & E.A. FOOT Biochem. J. **307** 707-712 (1995).

0022-3565/98/2872-0697$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 287:697–704, 1998

Vol. 287, No. 2
Printed in U.S.A.

Acute and Chronic Effects of Capsaicin in Perfused Rat Muscle: The Role of Tachykinins and Calcitonin Gene-Related Peptide[1]

CORY D. GRIFFITHS, DOMINIC P. GERAGHTY,[2] TRISTRAM P.D. ELDERSHAW and ERIC Q. COLQUHOUN

Division of Biochemistry, University of Tasmania, Hobart, Australia

Accepted for publication June 10, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

In perfused rat skeletal muscle (hindlimb), capsaicin either stimulates (submicromolar concentrations) or inhibits (micromolar concentrations) oxygen consumption (VO_2). Both VO_2 effects are associated with vasoconstriction, evident as an increase in perfusion pressure (PP), under constant flow. We have proposed that these effects are mediated by two vanilloid receptor subtypes: VN_1 (stimulation of VO_2) and VN_2 (inhibition of VO_2) (Colquhoun *et al.*, 1995; Griffiths *et al.*, 1996). In the present study, the role of capsaicin-sensitive neurons and sensory neuropeptides in the VN_1/VN_2 receptor actions of capsaicin was investigated. The observed maximum stimulation of VO_2 by capsaicin (0.4 μM; ΔVO_2, 1.35 ± 0.14 μmol g^{-1} h^{-1}) was accompanied by mild vasoconstriction (ΔPP, 5.8 ± 0.6 mm Hg). In contrast, 2 μM capsaicin produced strong inhibition of VO_2 (ΔVO_2, −2.25 ± 0.23 μmol g^{-1} h^{-1}) with pronounced vasoconstriction (ΔPP, 28.0 ± 1.3 mm Hg). VO_2 stimulation was significantly inhibited ($P < .05$) by the selective NK1 receptor antagonist CP-99994 (1 μM) and the NK2 receptor antagonist SR 48968 (1 μM) (by 42% and 51%, respectively), but PP was not altered. Infused SP and neurokinin A (NKA) stimulated VO_2 (observed maximum ΔVO_2, 0.52 ± 0.06 and 0.53 ± 0.08 μmol g^{-1} h^{-1}, respectively; EC_{50} values, 269 ± 23 and 21.2 ± 3.0 nM, respectively) and induced mild vasoconstriction (4.30 ± 0.33 and 6.75 ± 1.18 mm Hg, respectively; EC_{50} values, 352 ± 25.7 and 25.5 ± 2.7 nM, respectively). Neurokinin B (NKB) also stimulated VO_2 (maximum not determined) and vasoconstriction (maximum ΔPP, 3.40 ± 0.25 mm Hg; EC_{50}, 34.4 ± 5.2 nM). The rank order of potency for the tachykinins in this preparation was NKA > NKB > SP, which suggests stimulation primarily of NK2 receptors. Although infused calcitonin gene-related peptide (CGRP) did not alter hindlimb VO_2 or PP, the selective CGRP antagonist $CGRP_{(8-37)}$ markedly potentiated the inhibition of VO_2 produced by 1 μM capsaicin (84%) and the maximum capsaicin-induced vasoconstriction (57%), which indicates that endogenously released CGRP may act as a vasodilator. Hindlimbs perfused 1 day after capsaicin pretreatment showed attenuation of capsaicin-induced (0.4 μM) stimulation of VO_2 (92%) ($P < .05$) and vasoconstriction (64%), but this returned to normal after 7 days. The inhibition of VO_2 by 1 μM capsaicin was significantly ($P < .05$) enhanced 7 and 14 days after pretreatment (66% and 140%, respectively), as was the maximum vasoconstriction (64% and 68%, respectively). These data suggest that capsaicin-sensitive neurons, presumably *via* release of SP and NKA, are involved in VN_1 responses and that capsaicin pretreatment potentiates VN_2 responses, either by depletion of CGRP reserves or by up-regulation of putative VN_2 receptors.

The vanilloid spice principle capsaicin and its structural analogs (dihydrocapsaicin, resiniferatoxin, piperine, gingerols and shogaols) produce concentration-dependent vasoconstriction and a biphasic effect on skeletal muscle VO_2 in the constant-flow perfused rat hindlimb (Cameron-Smith *et al.*, 1990; Eldershaw *et al.*, 1992; Eldershaw *et al.*, 1994). Work from this laboratory suggests that the dual effect of vanilloids on VO_2 (stimulation and inhibition at low and high capsaicin concentrations, respectively) is mediated by at least two vanilloid receptor subtypes, designated VN_1 (stimulation of VO_2) and VN_2 (inhibition of VO_2) (Colquhoun *et al.*, 1995). This dual receptor hypothesis has recently been strengthened by the inhibition of the opposing VO_2 responses by selective competitive and noncompetitive vanilloid antagonists (Griffiths *et al.*, 1996). The putative VN_1 receptor appears to have a higher affinity for capsaicin and is more susceptible to blockade by capsazepine, a known competitive vanilloid antagonist (Urban and Dray, 1991; Bevan *et al.*, 1992). On the other hand, the VN_2 receptor has low affinity for capsaicin and capsazepine but is particularly sensitive to ruthenium red, a selective functional capsaicin antagonist at submicromolar concentrations (Amann and Maggi, 1991).

Although our previous findings show that the dual effects

Received for publication December 19, 1997.
[1] This work was supported in part by the National Health and Medical Research Council of Australia and the Australian Research Council.
[2] Present address: Department of Biomedical Science, University of Tasmania, Launceston, Australia 7250.

ABBREVIATIONS: SP, substance P; NKA, neurokinin A; NKB, neurokinin B; CGRP, calcitonin gene-related peptide; VO_2, oxygen consumption; PO_2, partial pressure of oxygen; PP, perfusion pressure; BSA, bovine serum albumin; EC_{50}, 50% of maximum response.

of capsaicin in perfused muscle are likely to be mediated by vanilloid receptor subtypes, the underlying mechanisms by which VN_1 and VN_2 receptors produce these responses are unknown. In other tissues, vanilloid receptors are thought to be coupled to nonselective cation channels on certain C-type and Aδ-type sensory neurons (James *et al.*, 1993). In fact, the recent cloning of a capsaicin receptor from dorsal root ganglia has revealed a 95-kD ion channel that is structurally related to members of the transient receptor potential (TRP) family of ion channels (Caterina *et al.*, 1997). Stimulation of these receptors facilitates the co-release of several neuropeptide transmitters, including the tachykinins SP and NKA, and CGRP (reviewed by Holzer, 1991). A hallmark of capsaicin action on peptide-containing neurons is its ability to induce a refractory state of sensory neuron block with prolonged or repeated *in vitro* application or after systemic administration (reviewed by Szolcsanyi, 1993).

Sensory neuropeptides released by capsaicin may produce a variety of biological responses, including changes in vascular tone and permeability, smooth muscle contraction, and inflammation (reviewed by Holzer, 1991). The actions of tachykinins are mediated by at least three receptor subtypes: SP-preferring NK1, NKA-preferring NK2 and NKB-preferring NK3 receptors (reviewed by Mussap *et al.*, 1993; Maggi *et al.*, 1993; Regoli *et al.*, 1994). These receptor preferences were originally based on the rank orders of potency of endogenous agonists, although each of the tachykinins will stimulate all three receptor types with varying affinity (Regoli *et al.*, 1994). NK1 receptors are widely distributed in both the CNS and peripheral tissues, whereas NK2 receptors are found mainly in peripheral tissues (predominantly on smooth muscle) and NK3 receptors in the CNS, although the latter are expressed in the rat portal vein and guinea pig myenteric plexus (Mastrangelo *et al.*, 1987; Guard *et al.*, 1990). At present there is little evidence for the presence of tachykinin receptors in skeletal muscle cells or skeletal muscle vasculature, although SP dilates the rat cremaster vasculature by a mechanism that is believed to involve the stimulation of NK1 receptors (Brock and Joshua, 1991), and vasodilation induced by stimulation of the rabbit tenuissimus muscle nerve is blocked by the SP antagonist spantide (Persson *et al.*, 1991).

Receptors for CGRP are tentatively divided into two distinct subtypes ($CGRP_1$ and $CGRP_2$) on the basis of the differing ability of C-terminal fragments of the peptide to antagonize the actions of intact CGRP in different preparations (reviewed by Poyner, 1995). CGRP receptors are expressed in cultured L6 rat skeletal muscle cells (Kreutter *et al.*, 1989; Poyner *et al.*, 1992) and whole rat skeletal muscle (Popper and Micevych, 1989; Pittner *et al.*, 1996). In addition, capsaicin has been shown to elicit vasodilation in a rat skeletal muscle preparation (cremaster) by stimulating the endogenous release of CGRP (White *et al.*, 1993).

The present study attempts to define a role for SP, NKA and CGRP in capsaicin-induced responses in the perfused hindlimb by 1) employing competitive NK1, NK2 and CGRP receptor antagonists (CP-99994, SR 48968 and $CGRP_{(8-37)}$), 2) examining the effects of SP, NKA, NKB and CGRP infusion and 3) examining the role of peptide-containing sensory neurons by investigating the effects of capsaicin pretreatment on hindlimb responses to infused capsaicin.

Materials and Methods

Rat hindlimb perfusion. All experimental procedures used in this study were approved by the University of Tasmania Animal Ethics Committee under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Australian Government Publishing Service, 1990).

Male Hooded-Wistar rats weighing 180 to 200 g were housed at 21 ± 1°C under a 12 h:12 h light:dark cycle and fed a commercial rat chow diet containing 21.4% protein, 4.6% lipid, 68% carbohydrate and 6% crude fiber with added vitamins and minerals. Water was supplied *ad libitum*.

Animals were anesthetized with pentobarbitone sodium (60 mg/kg) and their left hindlimbs perfused according to the method described previously (Colquhoun *et al.*, 1988). In brief, flow was isolated to the left hindlimb by cannulation of the abdominal aorta, posterior to the renal vessels, and ligation of the tail, right common iliac and cutaneous blood vessels. The hindlimb was perfused under constant-flow conditions (4.0 ± 0.1 ml/min) with a modified Krebs-Ringer bicarbonate buffer containing 8.3 mM glucose, 1.27 mM $CaCl_2$ and 2% BSA (fraction V) as an essential oncotic agent. All perfusions were conducted at 25°C, and the perfusate was continuously gassed with carbogen (95% O_2/5% CO_2) to ensure a constant arterial PO_2. The oxygen content of the venous effluent was measured continuously by directing outflow from the cannulated vena cava through an in-line 0.5-ml Clark-type oxygen electrode. PP was monitored by means of a pressure transducer adjoining the cannulated abdominal aorta.

The method of calculation of VO_2 has been described previously (Colquhoun *et al.*, 1988). Values for VO_2 calculation and perfusion pressure were taken only after steady-state conditions were obtained either under basal or drug-induced changes.

Agent infusion. Neuropeptides were dissolved into 20-μl aliquots using a 0.01 M acetic acid solution containing 1% β-mercaptoethanol and stored at −20°C to maintain chemical stability. The aliquots were then diluted, as needed, with 0.9% NaCl so that the acetate and β-mercaptoethanol concentrations were negligible. The neutral endopeptidase inhibitor phosphoramidon (5 μM) was co-infused with each neuropeptide (after the infusion of phosphoramidon alone for 5 min) to prevent enzymatic degradation. Because of the lipophilic nature of capsaicin, it was dissolved in 50% ethanol; thus care was taken to keep the infusion rates low (usually below 10 μl/min) to avoid vehicular perturbation. All other agents were dissolved in 0.9% saline. Capsaicin and the neuropeptides were infused with a syringe pump (Model 2620, Harvard Apparatus Inc., South Natick, MA) driving a 1.0-ml glass syringe (SGE, Australia) equipped with Teflon tubing. Other agents were infused with similar infusion pumps (Model 355, Sage Instruments, Orion Research Inc., (Beverly, MA or Model 11 microinfusion, Harvard Apparatus Inc.) also with an identical 1.0-ml glass syringe and Teflon tubing. All glass apparatus was silanized with Sigmacote before infusion to prevent peptide adhesion to glass surfaces.

In perfusions wherein CP-99994, SR 48968 or $CGRP_{(8-37)}$ was used, a control dose-response curve was first obtained by the cumulative infusion of increasing concentrations of capsaicin, followed by a period of recovery after drug removal. After re-establishment of basal VO_2 and PP, we infused CP-99994, SR 48968 or $CGRP_{(8-37)}$ alone for approximately 5 min, and then co-infused the antagonist while the capsaicin dose-response curve was repeated. When infused alone, none of the antagonists induced detectable changes in either basal VO_2 or PP.

Capsaicin pretreatment. Desensitization to capsaicin was induced by the method used previously by Cui and Himms-Hagen (1992), with a minor modification to the anesthetic used. Briefly, a total dose of 125 mg/kg capsaicin was administered, under anesthesia (40–60 mg/kg pentobarbitone), in four s.c. injections over a 3-day period (day 1, 12.5 mg/kg; day 2, 2 × 25 mg/kg; day 3, 62.5 mg/kg). Injections were given behind the neck or near the rump where s.c.

injection is easier because of the loose skin at these locations. Injections of the vehicle (10% Tween 80, 10% ethanol in normal saline) were given to control animals. The hindlimbs of all animals were perfused 1, 7 or 14 days after the final capsaicin (or vehicle) injection, and the responses to the infusion of the vanilloid were recorded.

Drugs and chemicals. SP, NKA, NKB, CGRP and $CGRP_{8-37}$ were purchased from Auspep (Australia); capsaicin, Sigmacote and phosphoramidon from the Sigma Chemical Company; BSA serum albumin (fraction V) from Boehringer Mannheim (Australia) and pentobarbitone sodium (Nembutal, 60 mg/ml) from Bomac Laboratories (Australia). Nonpeptide tachykinin antagonists were generous gifts: (2S,3S)-3-(2-methoxybenzyl)amino-2-phenylpiperidine (CP-99994) from Dr. S.B. Kadin, Pfizer Inc., Groton, CT, and (S)-N-methyl-N-[4-(4-acetylamino-4-phenyl piperidino)-2-(3,4dichlorophenyl) butyl]benzamide (SR 48968) from Dr. X. Emonds-Alt, Sanofi Recherche, Montpellier, France. All other reagents were of analytical grade.

Data analysis. Statistical analysis was performed by one-way analysis of variance (ANOVA) or ANOVA on ranks (Kruskal-Wallis analysis) where applicable. Paired data were analyzed by one-way repeated measures ANOVA or repeated measures ANOVA on ranks (Friedman analysis) where applicable. All ANOVAs were followed by multiple comparisons using the Student-Newman-Keuls method. $P < .05$ was considered statistically significant. The EC_{50} and E_{max} values for SP, NKA and NKB were estimated from VO_2 and PP concentration-response curves for individual experiments. For NKB, the maximum VO_2 effect was not obtained, so the EC_{50} for this peptide was estimated by using the mean E_{max} from the SP and NKA experiments. In capsaicin pretreatment experiments, the EC_{50} for the acute effects of capsaicin was estimated from individual PP concentration-response curves and statistically analyzed by Student's *t* test.

Results

Effects of CP-99994. Concentration-response curves for capsaicin were characteristically biphasic for VO_2, as seen previously (Colquhoun *et al.*, 1995; Griffiths *et al.*, 1996), with a concentration-dependent increase in PP that is indicative of vasoconstriction (fig. 1). Two consecutive concentration-response curves for capsaicin obtained in the same perfusion were very similar, as indicated by the data obtained using a low, ineffective concentration of CP-99994 (0.1 μM) (fig. 1A, D). However, there is occasionally mild sensitization to the VO_2 stimulatory response at a low capsaicin concentration (0.25 μM) (fig. 1B) that also occurs when a capsaicin dose-response curve is repeated in the absence of other agents (data not shown). The basis for this sensitization is unknown at present, but it may reflect an increase in the VN_1 receptor population or mild up-regulation of postreceptor cellular mechanisms. The observed maximum stimulation of VO_2 was produced by 0.4 μM capsaicin (ΔVO_2, 1.35 ± 0.14 μmol g^{-1} h^{-1} above basal VO_2) followed by inhibition of VO_2 at concentrations above 1 μM, maximum inhibition occurring at 2 μM (−2.25 ± 0.35 μmol g^{-1} h^{-1} below basal VO_2; fig. 1C). The nonpeptide NK1 receptor antagonist CP-99994 (0.5 and 1 μM) selectively inhibited the stimulation of VO_2 produced by capsaicin (ΔVO_2, 0.97 ± 0.03 and 0.78 ± 0.06 μmol g^{-1} h^{-1}, respectively, $P < .05$; fig. 1B, C). Some statistically significant differences in capsaicin-induced PP changes were observed in the presence of CP 99994 (fig. 1, D, E, F), but these were not consistent over the three antagonist concentrations used.

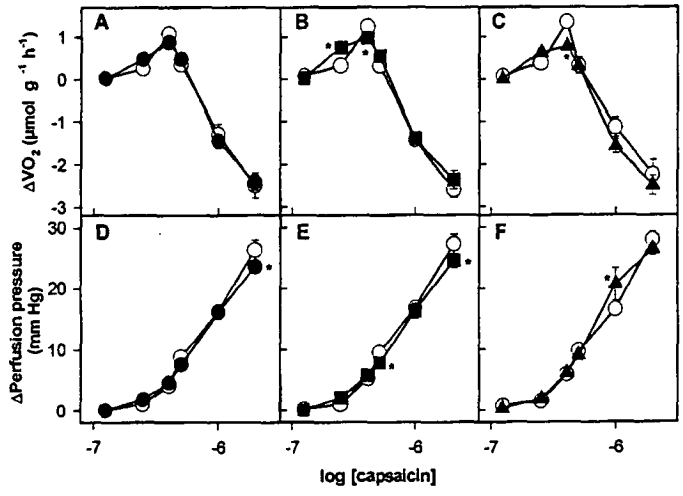

Fig. 1. Effect of the NK1 receptor antagonist CP-99994 on concentration-response curves for capsaicin-induced changes in oxygen consumption (panels A, B and C) and perfusion pressure (panels D, E and F) in the perfused rat hindlimb. Control (○), 0.1 μM (●), 0.5 μM (■) and 1.0 μM (▲) CP-99994. Statistical analysis was by one-way repeated measures ANOVA or repeated measures ANOVA on ranks (Friedman analysis) where applicable, followed by multiple comparisons (Student-Newman-Keuls method). * $P < .05$ from control. Values are mean ± S.E.M. of 5 to 6 experiments.

Effects of SR 48968. Consecutive concentration-response curves for capsaicin were very similar at an ineffective concentration of the selective NK2 receptor antagonist SR 48968 (fig. 2, A and D), a result that confirms the reproducibility of capsaicin-induced effects. At a concentration of 1 μM, SR 48968 significantly inhibited ($P < .05$) the maximum stimulation of VO_2 induced by 0.4 μM capsaicin (ΔVO_2: control, 1.06 ± 0.13 μmol g^{-1} h^{-1}; SR 48968, 0.52 ± 0.24 μmol g^{-1} h^{-1}; fig. 2B). Although the stimulation of VO_2 at a lower concentration of capsaicin (0.25 μM) was potentiated in the presence of 1 μM SR 48968 (fig. 2B), this effect is likely to be caused not by the antagonist, but rather by the mild sensitization to capsaicin that occurs when doses of the vanilloid are repeated in a single perfusion (see above). Furthermore, there was not a statistically significant difference in the VO_2 response to 0.25 μM capsaicin when a higher concentration of SR 48968 (10 μM) was used (fig. 2C). However, at this concentration of SR 48968, further blockade of the maximum capsaicin-induced stimulation of VO_2 (ΔVO_2: control, 1.03 ± 0.08 μmol g^{-1} h^{-1}; SR 48968, 0.17 ± 0.30 μmol g^{-1} h^{-1}, $P < .05$; fig. 2C) was evident, whereas the inhibition of VO_2 produced by a high concentration of the vanilloid (2 μM) was significantly enhanced (ΔVO_2: control, −2.07 ± 0.20 μmol g^{-1} h^{-1}; SR 48968, −3.04 ± 0.26 μmol g^{-1} h^{-1}, $P < .05$). Vasoconstriction at all concentrations of capsaicin was also significantly ($P < .05$) enhanced by 10 μM SR 48968 (fig. 2F).

Effects of $CGRP_{(8-37)}$. Infusion of the CGRP antagonist $CGRP_{(8-37)}$ significantly ($P < .05$) increased the stimulation of VO_2 induced by 0.25 μM capsaicin (ΔVO_2: control, 0.13 ± 0.06 μmol g^{-1} h^{-1}; $CGRP_{(8-37)}$, 0.80 ± 0.09 μmol g^{-1} h^{-1}) but did not significantly increase the observed maximum stimulation of VO_2 produced by the infusion of 0.4 μM capsaicin (fig. 3A). The inhibition of VO_2 induced by 1 μM capsaicin was significantly enhanced by the co-infusion of $CGRP_{(8-37)}$ (ΔVO_2: control, −1.13 ± 0.29 μmol g^{-1} h^{-1}; $CGRP_{(8-37)}$, −2.08 ± 0.15 μmol g^{-1} h^{-1}, $P < .05$, fig. 3A), whereas ΔPP at 1 and 2 μM capsaicin was markedly increased (ΔPP: control, 16.5 ± 0.7 mm Hg and 29.3 ± 2.0 mm

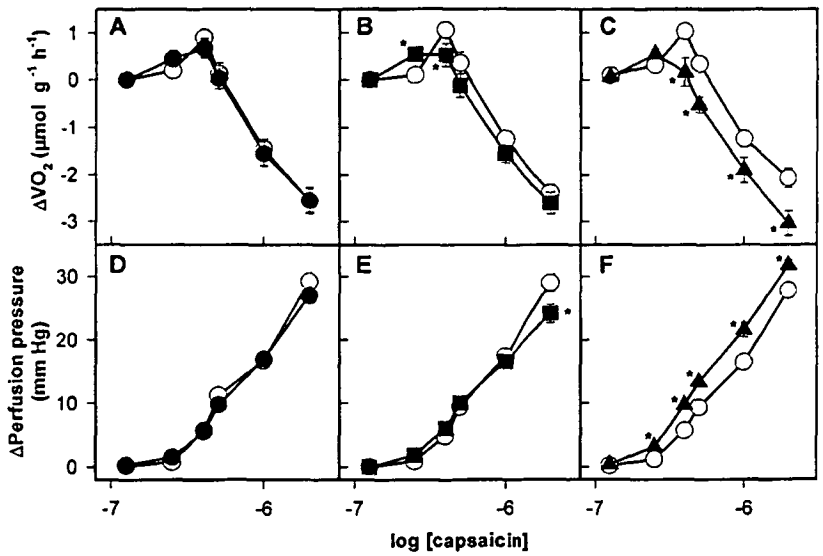

Fig. 2. Effect of the NK2 receptor antagonist SR 48968 on concentration-response curves for capsaicin-induced changes in oxygen consumption (panels A, B and C) and perfusion pressure (panels D, E and F) in the perfused rat hindlimb. Control (○), 0.1 μM (●), 1.0 μM (■) and 10.0 μM (▲) SR48968. Statistical analysis was by one-way repeated measures ANOVA or repeated measures ANOVA on ranks (Friedman analysis) where applicable, followed by multiple comparisons (Student-Newman-Keuls method). * $P < .05$ from control. Values are mean ± S.E.M. of 5 to 6 experiments.

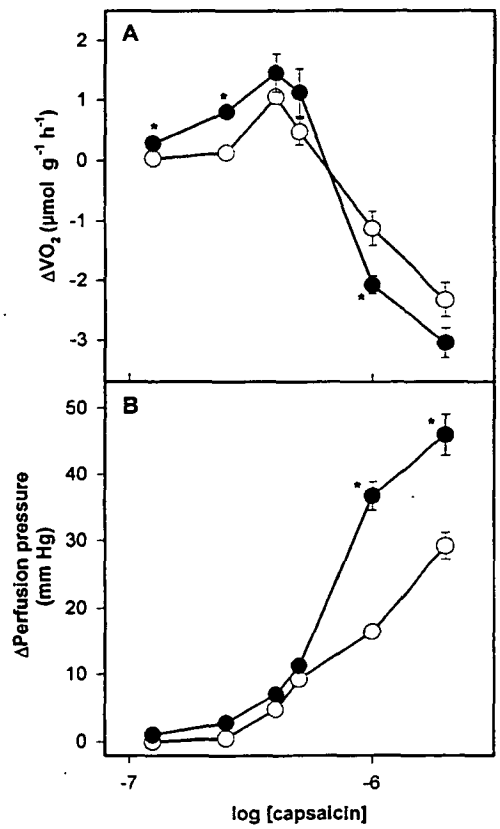

Fig. 3. Effect of the CGRP receptor antagonist $CGRP_{(8-37)}$ on concentration-response curves for capsaicin-induced changes in oxygen consumption (panel A) and perfusion pressure (panel B) in the perfused rat hindlimb. Control (○) and 1.0 μM $CGRP_{(8-37)}$ (●). Statistical analysis was by one-way repeated measures ANOVA or repeated measures ANOVA on ranks (Friedman analysis) where applicable, followed by multiple comparisons (Student-Newman-Keuls method). * $P < .05$ from control. Values are mean ± S.E.M. of 5 to 6 experiments.

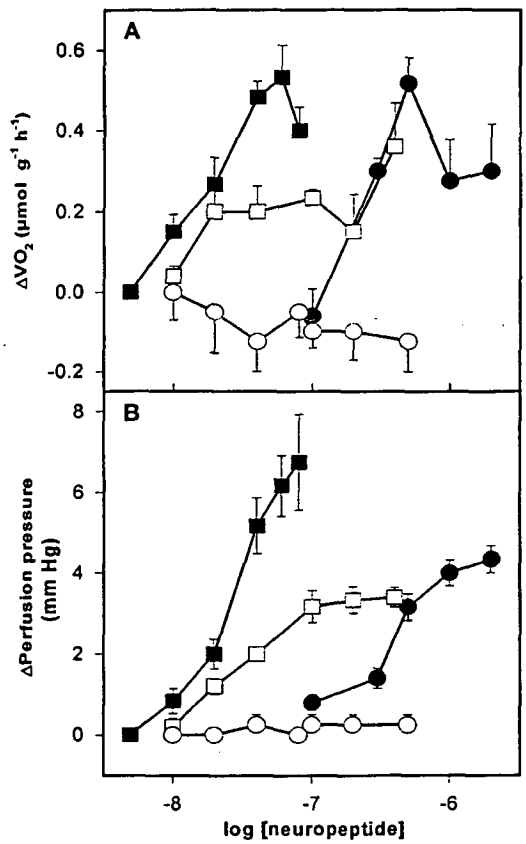

Fig. 4. Effect of SP (●), NKA (■), NKB (□) and CGRP (○) on oxygen consumption (panel A) and perfusion pressure (panel B) in the perfused rat hindlimb. In all experiments, SP, NKA, NKB and CGRP were co-infused with the neutral endopeptidase inhibitor phosphoramidon (5 μM). Values are mean ± S.E.M. of 4 to 6 experiments.

Hg, respectively; $CGRP_{(8-37)}$, 36.8 ± 2.1 mm Hg and 46.0 ± 3.1 mm Hg, respectively, $P < .05$).

Effects of SP, NKA, NKB and CGRP. Infusion of the neutral endopeptidase inhibitor phosphoramidon (5 μM) alone had no detectable effect on either basal VO_2 or PP. The co-infusion of increasing doses of SP with phosphoramidon produced a concentration-dependent increase in VO_2 (fig. 4A; table 1) and induced mild vasoconstriction (fig. 4B; table 1). Increasing the dose of SP to micromolar concentrations caused some attenuation of the VO_2 increase, whereas the effect on PP plateaued. NKA, also co-infused with phosphoramidon, produced similar effects on hindlimb VO_2 and PP but was approximately 10-fold more potent than SP (fig. 4; table 1). The infusion of NKB, with phosphoramidon, stimu-

TABLE 1
Maximum change in perfusion pressure (ΔPP) and oxygen consumption (ΔVO_2), and concentration producing 50 percent of maximum response (EC_{50}) for SP, NKA and NKB in the perfused rat hindlimb

Neuropeptide	n	ΔPP		ΔVO_2	
		E_{max} (mm Hg)	EC_{50} (nM)	E_{max} (μmol g^{-1} h^{-1})	EC_{50} (nM)
SP	5	4.33 ± 0.33	352 ± 26	0.52 ± 0.07	269 ± 23
NKA	4	6.75 ± 1.18	25.5 ± 2.7	0.53 ± 0.08	21.2 ± 3.0
NKB	5	3.40 ± 0.25	34.4 ± 5.2	—	71.8 ± 29.2†

Values are mean ± S.E.M.
† EC_{50} estimated using mean of E_{max} (ΔVO_2) for SP and NKA.

lated a small but reproducible change in VO_2; however, maximum VO_2 was not obtained using concentrations of NKB that induced a maximum change in vascular tone (fig. 4; table 1). On the other hand, the co-infusion of CGRP (10–500 nM) and phosphoramidon altered neither basal hindlimb VO_2 nor vascular tension.

Effects of capsaicin pretreatment. Figure 5 shows VO_2 and PP responses to capsaicin in hindlimbs perfused 1, 7 and 14 days after vehicle or systemic capsaicin pretreatment. The stimulation of VO_2 induced by submicromolar concentrations of capsaicin was significantly inhibited 1 day after capsaicin pretreatment (maximum ΔVO_2: control, 0.98 ± 0.23 μmol g^{-1} h^{-1}; capsaicin-pretreated, 0.08 ± 0.04 μmol g^{-1} h^{-1}, $P < .05$; fig. 5A), whereas the increase in PP produced by 2 μM capsaicin was markedly enhanced (ΔPP: control, 23.2 ± 1.4 mm Hg; capsaicin-pretreated, 35.8 ± 3.3 mm Hg, $P < .05$; fig. 5D). Seven and 14 days after capsaicin pretreatment, the stimulation of VO_2 and the vasoconstriction induced by low concentrations of capsaicin was completely restored, whereas the maximum inhibition of VO_2 by 2 μM capsaicin was significantly enhanced compared with vehicle-pretreated controls (ΔVO_2: 7 days, control, −3.18 ± 0.06, capsaicin-pretreated, −4.27 ± 0.46; 14 days, control, −3.02 ± 0.25, capsaicin-pretreated, −4.52 ± 0.40 μmol g^{-1} h^{-1}; fig. 3B, C). The maximum vasoconstriction at micromolar concentrations of capsaicin was also greatly increased 7 days after capsaicin pretreatment, and it was increased further after 14

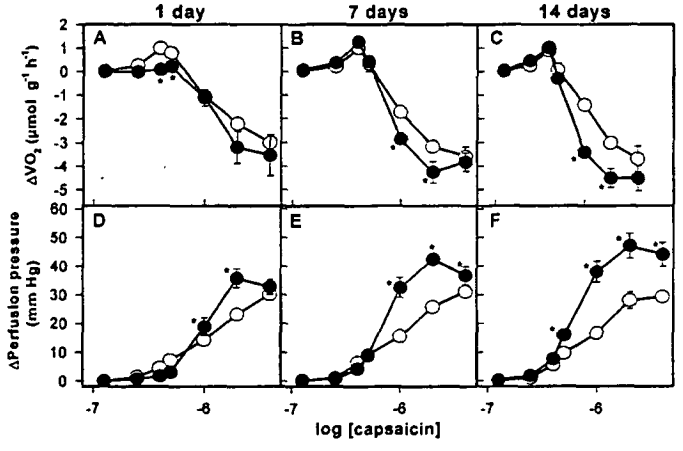

Fig. 5. Concentration-response curves for capsaicin-induced changes in oxygen consumption (panels A, B and C) and perfusion pressure (panels D, E and F) in the hindlimbs of rats perfused 1, 7 and 14 days after pretreatment with vehicle (○) or capsaicin (●). Statistical analysis was by one-way ANOVA or ANOVA on ranks (Kruskal-Wallis analysis) where applicable, followed by multiple comparisons (Student-Newman-Keuls method). * $P < .05$ from control. Values are mean ± S.E.M. of 4 to 6 experiments.

TABLE 2
Maximum change in perfusion pressure (ΔPP) and concentration producing 50 percent of maximum response (EC_{50}) for capsaicin in the perfused rat hindlimb, 1, 7 and 14 days after vehicle- or capsaicin-pretreatment

Pretreatment	n	Days after Pretreatment	E_{max} (mm Hg)	EC_{50} (μM)
Vehicle	5	1	30.2 ± 2.2	1.07 ± 0.04
Capsaicin	5	1	35.8 ± 3.3	0.99 ± 0.07
Vehicle	4	7	31.0 ± 1.8	1.02 ± 0.05
Capsaicin	4	7	42.3 ± 0.3	0.74 ± 0.04**
Vehicle	5	14	29.3 ± 1.8	0.96 ± 0.05
Capsaicin	5	14	47.0 ± 4.4	0.66 ± 0.05**

Values are mean ± S.E.M. ** $P < 0.01$ (Student's t test) from corresponding vehicle pretreated.

days (fig. 5; table 2). In addition, the EC_{50} for capsaicin, estimated from the PP concentration-response curves, was significantly ($P < .01$) lower in animals perfused 7 and 14 days after capsaicin pretreatment (table 2).

Discussion

Capsaicin produced a powerful vasoconstrictor response and a biphasic effect on VO_2 in the perfused rat hindlimb, a result that confirmed previous data from this laboratory (Cameron-Smith *et al.*, 1990; Colquhoun *et al.*, 1995; Griffiths *et al.*, 1996). The main purpose of the present study was to investigate the role of sensory neurons and sensory neuropeptides (SP, NKA, NKB and CGRP) in capsaicin-induced changes in vascular resistance and VO_2 by studying the effects of capsaicin pretreatment and neuropeptide antagonists.

Stimulation of VO_2 produced by submicromolar concentrations of capsaicin (VN_1 response) was partly blocked by the selective NK1 receptor antagonist CP-99994 in a concentration-dependent manner (fig. 1). The NK2 receptor antagonist SR 48968 produced effects similar to those of CP-99994 but also enhanced the inhibition of VO_2 produced by micromolar concentrations of capsaicin (VN_2 response) and potentiated vasoconstriction over the entire capsaicin concentration range (fig. 2). Infusion of SP, NKA or NKB, in the presence of phosphoramidon, produced mild, concentration-dependent vasoconstriction and stimulated VO_2 (fig. 4). NKA was at least 10-fold more potent than SP at stimulating VO_2 and vasoconstriction (table 1), and its activity is comparable to that in an NK2 receptor bioassay (rabbit pulmonary artery) (Regoli *et al.*, 1987). The potency of SP in the present study is at least 1000-fold lower than in the NK1 receptor bioassay (dog carotid artery) and more closely resembles its activity on NK2 receptors in the rabbit pulmonary artery (Regoli *et al.*, 1987). However, the use of BSA as an essential colloid in the perfused hindlimb preparation may account for the apparent low potency of SP; this protein is known to bind numerous agents, including capsaicin. Taken together, these findings using neuropeptide agonists and antagonists provide evidence that stimulation of VO_2 by submicromolar concentrations of capsaicin is partly mediated by the endogenous release of SP and NKA, which then stimulate VO_2 *via* action on peripheral NK2 receptors and possibly NK1 receptors. However, the data obtained using nonpeptide tachykinin receptor antagonists should be interpreted with caution, because the submicromolar to micromolar concentrations required to alter the effects of capsaicin may not be specific for one tachy-

kinin receptor subtype and may induce nonspecific effects (Lombet and Spedding, 1994). Nonetheless, when taken in conjunction with the rank order of potency for the tachykinins in this preparation (NKA > NKB > SP), the present data support the notion of NK2 receptor involvement, although a role for NK1 receptors cannot be excluded because CP-99994 was also effective at blocking some actions of capsaicin. In addition, NKA is known to have a strong affinity for NK1 receptors, and preliminary autoradiographic studies indicate that NK1 receptors are present on blood vessels in hindlimb skeletal muscle (Griffiths, Mazzone, Geraghty and Colquhoun, unpublished observations). Although NKB stimulated VO_2 and vasoconstriction in the present study, it is unlikely that NK3 receptors play a role in the capsaicin-mediated effects in muscle, because their peripheral distribution is limited (Mastrangelo *et al.*, 1987; Guard *et al.*, 1990).

The potentiation of capsaicin-stimulated vasoconstriction by SR 48968 may indicate that endogenously released tachykinins, acting *via* NK2 receptors, are dilators of the perfused hindlimb vasculature, although the concentration of SR 48968 required for this effect may have also blocked NK1 receptors. Similarly, CGRP, which is released in skeletal muscle in response to capsaicin (Santicioli *et al.*, 1992), may act as a potent vasodilator in this preparation, because the CGRP receptor antagonist $CGRP_{(8-37)}$ greatly potentiated the capsaicin-induced vasoconstriction and inhibition of VO_2 (fig. 3). These hypotheses are not supported by the infusion, in the presence of phosphoramidon, of the tachykinins SP and NKA, which act as mild vasoconstrictors in this preparation (see above). Infused CGRP (also with phosphoramidon) did not produce a measurable effect on basal hindlimb VO_2 or vascular tone (fig. 4). This observation is unusual, given that CGRP has been shown to be a potent vasodilator in many tissues, including striated muscle (White *et al.*, 1993; Kim *et al.*, 1995). In addition, it has recently been shown that CGRP, released from capsaicin-sensitive primary afferents, contributes to the hyperemic response to skeletal muscle contraction (*via* sciatic nerve stimulation) in the rat hindlimb (Yamada *et al.*, 1997a, b). However, basal hindlimb PP in the present study probably represents near-maximum arteriolar dilation, because at the flow rate used (4 ml/min), the potent vasodilator nitroprusside has no measurable effect on vascular tone (Colquhoun *et al.*, 1988; Ye *et al.*, 1990). This may limit the scope of action of SP, NKA, NKB and/or CGRP such that any vasodilator action by these peptides would not be observed. The vasoconstriction induced by SP, NKA and NKB in the present study may have resulted from direct stimulation of smooth muscle cell NK receptors after diffusion of the peptides across the endothelium. It remains to be seen whether the neuropeptides used in the present study can significantly alter vascular tone in the constant-flow perfused-hindlimb preparation preconstricted with other vasoactive agents (*e.g.*, norepinephrine, serotonin and angiotensin II). Preliminary results obtained in the perfused rat hindlimb under norepinephrine-induced vascular tension indicate that these peptides may induce vasodilation, although it is not yet clear which receptors and mechanisms are involved in this response (Griffiths, Geraghty and Colquhoun, unpublished observations).

Capsaicin possesses a well-documented ability to stimulate and then desensitize peptide-containing sensory neurons with prolonged or repeated application or after systemic administration. Indeed, capsaicin is a widely used research tool that selectively blocks C-type and Aδ-type primary afferents. In the present investigation, we attempted to define a role for capsaicin-sensitive neurons in the acute metabolic and vascular effects of vanilloids in perfused muscle by studying the effects of systemic capsaicin pretreatment. Capsaicin pretreatment produced dramatically alters capsaicin-induced VO_2 and PP changes in the perfused hindlimb (fig. 5). One day after capsaicin pretreatment, the stimulation of VO_2 and the mild increase in PP produced by submicromolar concentrations of capsaicin (VN_1 response) were almost completely abolished. However, 7 days after capsaicin pretreatment, the VN_1 response had returned, and the magnitude of VO_2 stimulation was identical to that of the control.

Szolcsanyi (1993) describes four distinct actions of capsaicin pretreatment on sensory neurons: 1) release of neuropeptides within minutes; 2) "sensory neuron block," wherein sensory neurons are unresponsive to capsaicin (*i.e.*, neuropeptides are not released), which lasts for hours to several days; 3) recovery of function of some neurons and degeneration of others over several days to weeks and 4) complete degeneration of affected neurons over weeks to months. In the present study, acute sensory neuron block may explain the absence of the VN_1 response 1 day after capsaicin pretreatment. The re-establishment of the VN_1 response after 7 days may be due to a small population of intact C fibers that recover from the block and release sufficient neuropeptides to stimulate VO_2.

In contrast to the effects of capsaicin pretreatment on VN_1 responses, the inhibition of VO_2 (VN_2 response) was marginally enhanced 1 day, and significantly enhanced 7 and 14 days, after capsaicin pretreatment. A progressive increase in the vasoconstrictor response to capsaicin mirrored the enhancement of VO_2 inhibition, the maximum PP to 2 μM capsaicin infusion almost doubling 14 days after capsaicin pretreatment. Further analysis of the data revealed that the concentration of capsaicin producing a half-maximal increase in PP was significantly ($P < .01$) decreased 7 and 14 days after capsaicin pretreatment. Why the maximum vasoconstrictor response progressively increased in capsaicin-pretreated rats is unclear. This was an unexpected finding because capsaicin pretreatment normally leads to a blunting of nonvascular, smooth muscle responses to capsaicin (Maggi and Meli, 1988). This observation, when combined with the decrease in EC_{50} for capsaicin, suggests either up-regulation of VN_2 receptors and/or sensitization of vascular smooth muscle to the direct constrictor action of capsaicin. Alternatively, the apparent increased sensitivity of the vasculature to constrict under capsaicin stimulation may be due to the absence of sufficient vasodilator peptides (*e.g.*, CGRP) to counteract the direct action of the vanilloid on vascular smooth muscle. In cats, "cold storage denervation" potentiates capsaicin-induced vasoconstriction of large cerebral arteries that correlates with degeneration of SP- and CGRP-containing perivascular nerves (Saito *et al.*, 1988). These authors suggested that although capsaicin releases vasodilator peptides (presumably SP, CGRP, etc.) from perivascular nerves of cat cerebral arteries, a direct vasoconstrictor effect of capsaicin predominates. This hypothesis is supported by the work of Edvinsson *et al.* (1990), who showed that the vasodilatation induced by capsaicin in cat cerebral arteries

was attenuated by repeated capsaicin application or by trigeminal ganglionectomy, whereas the vasoconstrictor effect was unaltered. Similarly, Duckles (1986) has shown that capsaicin applied to the isolated carotid artery and thoracic aorta of the guinea pig causes vasoconstriction, rather than dilation, after systemic *in vivo* capsaicin pretreatment. The apparent direct vasoconstrictor action observed in this study is also believed to be due to the absence of sufficient sensory vasodilator peptides after capsaicin pretreatment. However, the studies of Saito *et al.* (1988), Edvinsson *et al.* (1990) and Duckles (1986) suggest that the vasoconstrictor action of capsaicin occurs by a nonspecific effect on the plasma membrane of vascular smooth muscle cells. Conversely, the effects in the perfused hindlimb are believed to occur *via* the stimulation of specific vanilloid receptors because the vasoconstriction can be blocked by the competitive vanilloid receptor antagonist capsazepine (Griffiths *et al.*, 1996).

Exactly how capsaicin and the sensory neuropeptides produce their vascular and VO_2 effects in perfused muscle is unclear. The concept of site-specific vasoconstriction, leading to increased "nutritive" flow, has been proposed to explain the large increases in hindlimb VO_2 seen with the infusion of other potent vasoconstrictors, such as norepinephrine, angiotensin II and vasopressin (reviewed in Clark *et al.*, 1995; 1997). That is, vasoconstrictors that increase hindlimb VO_2 probably do so by redistributing perfusate flow to the network of vessels supplying skeletal muscle cells, which results in greater total nutrient exchange. On the basis of this flow redistribution model, it appears plausible that submicromolar concentrations of capsaicin may stimulate VO_2 (VN_1 response) by selectively constricting (*via* a direct effect) or dilating (by release of neuropeptides) blood vessels, leading to increased perfusate flow to "nutritive" vessels. However, a direct effect of capsaicin and the sensory neuropeptides to stimulate muscle VO_2 cannot be ruled out, because in the present study, NK1 and NK2 receptor antagonists decreased capsaicin-induced stimulation of VO_2 but did not cause appreciable changes in PP (fig. 1C, F; fig. 2, B and E).

On the other hand, there is convincing evidence that strong vasoconstrictors that inhibit VO_2 in the perfused hindlimb (*e.g.*, serotonin) do so by shunting perfusate away from nutritive vessels to non-nutritive vessels supplying hindlimb connective tissue (septa and tendons) (Newman *et al.*, 1997). Therefore, increased non-nutritive flow may explain the inhibition of VO_2 that accompanies the strong vasoconstriction induced by high concentrations of capsaicin. This hypothesis is strengthened by the current observation that the augmentation of capsaicin induced vasoconstriction 7 and 14 days after capsaicin pretreatment (fig. 5) produced a concomitant potentiation of VO_2 inhibition.

The results of the present study imply that capsaicin, when infused into the perfused rat hindlimb, stimulates higher-affinity vanilloid receptors (VN_1) that release thermogenic (VO_2-stimulating) peptides. These receptors appear to be neuronal (primary afferent C fiber), given that systemic capsaicin pretreatment ablates the acute VO_2 stimulation response to infused capsaicin. The stimulation of VO_2 by capsaicin is also selectively blocked by nonpeptide tachykinin antagonists of NK1 and NK2 receptors, and infused SP, NKA and NKB stimulate oxygen consumption and mild vasoconstriction with a rank potency order of NKA > NKB > SP. Hence, capsaicin may stimulate VO_2 by releasing endogenous tachykinins that interact primarily with NK2 receptors. Conversely, CGRP had no detectable effect on VO_2 or pressure, which may be due to the use of an almost fully dilated preparation. Indeed, the CGRP antagonist $CGRP_{(8-37)}$ enhanced capsaicin-induced vasoconstriction and inhibition of VO_2, which suggests that a direct vasoconstrictor action of capsaicin is opposed by the vasodilator action of CGRP. Consequently, the enhanced vasoconstrictor response to capsaicin in capsaicin-pretreated rats (7 and 14 days) may be due to a reduction in the release of CGRP from sensory neurons. Thus in the perfused rat hindlimb, the overall degree of capsaicin-induced vasoconstriction may be the sum of the indirect actions of vasoactive peptides (*e.g.*, SP, NKA and CGRP) released from sensory neurons, plus the direct vasoconstrictor action of capsaicin on vascular smooth muscle.

Acknowledgments

The authors wish to thank Dr. Jiming Ye for his assistance with the preparation of this paper. We also extend our thanks to Dr. S.B. Kadin (Pfizer Inc.) and Dr. X. Emonds-Alt (Sanofi Recherche) for their generous gifts of CP-99994 and SR 48968, respectively.

References

Amann R and Maggi CA (1991) Ruthenium red as a capsaicin antagonist. *Life Sci* **49**:849–856.

Australian Government (1990) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Australian Government Publishing Service, Canberra.

Bevan S, Hothi S, Hughes G, James IF, Rang HP, Shah K, Walpole CSJ and Yeats JC (1992) Capsazepine: A competitive antagonist of the sensory neuron excitant capsaicin. *Br J Pharmacol* **107**:544–552.

Brock JW and Joshua IG (1991) Substance P mechanisms in the regulation of striated muscle microcirculation. *Microvasc Res* **41**:24–28.

Cameron-Smith D, Colquhoun EQ, Ye J-M, Hettiarachchi M and Clark MG (1990) Capsaicin and dihydrocapsaicin stimulate oxygen consumption in the perfused rat hindlimb. *Int J Obesity* **14**:259–270.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD and Julius D (1997) The capsaicin receptor: A heat-activated ion channel in the pain pathway. *Nature (Lond)* **389**:816–824.

Clark MG, Colquhoun EQ, Rattigan S, Dora KA, Eldershaw TPD, Hall JL and Ye J (1995) Vascular and endocrine control of muscle metabolism. *Am J Physiol* **268** (*Endocrinol Metab* 31):E797–E812.

Clark MG, Rattigan S, Dora KA, Newman JMB, Steen JT, Miller KA and Vincent MA (1997) Vascular and metabolic regulation of muscle, in *Physiology, Stress and Malnutrition: Functional Correlates, Nutritional Intervention* Kinney JM and Tucker HN (eds), pp 325–346, Lippincott-Raven Publishers, New York.

Colquhoun EQ, Hettiarachchi M, Ye J-M, Richter EA, Hniat AJ, Rattigan S and Clark MG (1988) Vasopressin and angiotensin II stimulate oxygen uptake in the perfused rat hindlimb. *Life Sci* **43**:1747–1754.

Colquhoun EQ, Eldershaw TPD, Bennett KL, Hall JL, Dora KA and Clark MG (1995) Functional and metabolic evidence for two different vanilloid (VN_1 and VN_2) receptors in perfused rat hindlimb. *Life Sci* **57**:91–102.

Cui J and Himms-Hagen J (1992) Rapid but transient atrophy of brown adipose tissue in capsaicin-desensitised rats. *Am J Physiol* **262** (*Regulatory Integrative Comp Physiol* **31**):R562–R567.

Duckles SP (1986) Effects of capsaicin on vascular smooth muscle. *Naunyn-Schmiedeberg's Arch Pharmacol* **333**:59–64.

Edvinsson L, Jansen I, Kingman TA and McCulloch J (1990) Cerebrovascular responses to capsaicin *in vitro* and *in situ*. *Br J Pharmacol* **100**:312–318.

Eldershaw TPD, Colquhoun EQ, Dora KA, Peng Z-C and Clark MG (1992) Pungent principles of ginger (*Zingiber officinale*) are thermogenic in the perfused rat hindlimb. *Int J Obesity* **16**:755–763.

Eldershaw TPD, Dora KA, Bennett K, Clark MG and Colquhoun EQ (1994) Resiniferatoxin and piperine: Capsaicin-like stimulators of oxygen uptake in the perfused rat hindlimb. *Life Sci* **55**:389–397.

Griffiths CD, Eldershaw TPD, Geraghty DP, Hall JL and Colquhoun EQ (1996) Capsaicin-induced biphasic oxygen uptake in rat muscle: Antagonism by capsazepine and ruthenium red provides further evidence for peripheral vanilloid receptor subtypes (VN_1/VN_2). *Life Sci* **59**:105–117.

Guard S, Watson SP, Maggio JE, Too HP and Watling KJ (1990) Pharmacological analysis of [^{3}H]-senktide binding to NK3 tachykinin receptors in guinea-pig longitudinal muscle-myenteric plexus and cerebral cortex membranes. *Br J Pharmacol* **90**:767–773.

Holzer P (1991) Capsaicin: Cellular targets, mechanisms of action and selectivity for thin sensory neurons. *Pharmacol Rev* **42**:143–201.

James IF, Ninkina NN and Wood JN (1993) The capsaicin receptor, in *Capsaicin in the Study of Pain.* (Wood JN ed) pp 83–102, Academic Press Ltd, London.

Kim C, Roberts AM and Joshua IG (1995) Differences in the capsaicin-induced dilation of arterioles and venules in rat striated muscle. *J Pharmacol Exp Ther* **273**:605–610.

Kreutter D, Orena SJ and Andrew KM (1989) Suppression of insulin-stimulated

glucose transport in L6 myocytes by calcitonin gene-related peptide. *Biochem Biophys Res Commun* **164**:461–467.
Lombet A and Spedding M (1994) Differential effects of non-peptide tachykinin antagonists on Ca^{2+} channels. *Eur J Pharmacol* **267**:113–115.
Maggi CA and Meli A (1988) The sensory efferent function of capsaicin-sensitive sensory neurons. *Gen Pharmac* **19**:1–43.
Maggi CA, Patacchini R, Rovero P and Giachetti A (1993) Tachykinin receptors and tachykinin receptor antagonists. *J Auton Pharmacol* **13**:23–93.
Mastrangelo D, Mathison R, Huggel HJ, Dion S, D'Orleans-Juste P, Rhaleb N-E, Drapeau G, Rovero P and Regoli D (1987) The rat isolated portal vein: A preparation sensitive to neurokinins, particularly neurokinin B. *Eur J Pharmacol* **134**: 321–326.
Mussap CJ, Geraghty DP and Burcher E (1993) Tachykinin receptors: A radioligand binding perspective. *J Neurochem* **60**:1987–2009.
Newman JMB, Steen JT and Clark MG (1997) Vessels supplying septa and tendons as functional shunts in perfused rat hindlimb. *Microvasc Res* **54**:49–57.
Persson MG, Hedqvist P and Gustafsson LE (1991) Nerve-induced tachykinin-mediated vasodilatation in skeletal muscle is dependent on nitric oxide formation. *Eur J Pharmacol* **205**:295–301.
Pittner RA, Wolfe-Lopez D, Young AA and Beaumont K (1996) Different pharmacological characteristics in L6 and C_2C_{12} muscle cells and intact skeletal muscle for amylin, CGRP and calcitonin. *Br J Pharmacol* **117**:847–852.
Popper P and Micevych PE (1989) Localisation of calcitonin gene-related peptide and its receptors in a striated muscle. *Brain Res* **496**:180–186.
Poyner D (1995) Pharmacology of receptors for calcitonin gene-related peptide and amylin. *Trends Pharmacol Sci* **16**:424–428.
Poyner D, Andrew DP, Brown D, Bose C and Hanley M (1992) Pharmacological characterisation of a receptor for calcitonin gene-related peptide on rat, L6 myocytes. *Br J Pharmacol* **105**:441–447.
Regoli D, Drapeau G, Dion S and D'Orléans-Juste P (1987) Pharmacological receptors for substance P and neurokinins. *Life Sci* **40**:109–117.
Regoli D, Boudon A and Fauchere J-L (1994) Receptors and antagonists for substance P and related peptides. *Pharmacol Rev* **46**:551–599.
Saito A, Masaki T, Lee TJ-F and Goto K (1988) Effects of capsaicin on the contractility and peptide-containing nerves of large cerebral arteries of the cat. *Arch Int Pharmacodyn* **295**:194–203.
Santicioli P, Del Blanco E, Geppetti P and Maggi CA (1992) Release of calcitonin gene-related peptide-like (CGRP-LI) immunoreactivity from rat isolated soleus muscle by low pH, capsaicin and potassium. *Neurosci Lett* **143**:19–22.
Szolcsanyi J (1993) Actions of capsaicin on sensory receptors, in *Capsaicin in the Study of Pain.* (Wood JN ed), pp 1–27, Academic Press Ltd, London.
Urban L and Dray A (1991) Capsazepine, a novel capsaicin antagonist, selectively antagonises the effects of capsaicin in the mouse spinal cord *in vitro*. *Neurosci Lett* **134**:9–11.
White CB, Roberts AM and Joshua IG (1993) Arteriolar dilation mediated by capsaicin and calcitonin gene-related peptide in rats. *Am J Physiol* **265** (*Heart Circ Physiol* 34):H1411–1415.
Yamada M, Ishikawa T, Fujimori A and Goto K (1997a) Local neurogenic regulation of rat hindlimb circulation—role of calcitonin gene-related peptide in vasodilatation after skeletal muscle contraction. *Br J Pharmacol* **122**:703–709.
Yamada M, Ishikawa T, Yamanaka A, Fujimori A and Goto K (1997b) Local neurogenic regulation of rat hindlimb circulation—CO_2-induced release of calcitonin gene-related peptide from sensory nerves. *Br J Pharmacol* **122**:710–714.
Ye J-M, Colquhoun EQ, Hettiarachchi M and Clark MG (1990) Flow-induced oxygen uptake by the perfused rat hindlimb is inhibited by vasodilators and augmented by norepinephrine: A possible role for the microvasculature in hindlimb thermogenesis. *Can J Physiol Pharmacol* **68**:119–125.

Send reprint requests to: D.P. Geraghty, Department of Biomedical Science, University of Tasmania, Launceston, Australia 7250.