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

CORY D. GRIFFITHS, DOMINIC P. GERAGHTY, 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, piperoxin, gingenols 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 capsacain antagonist at submicromolar concentrations (Amann and Maggi, 1991).

Although our previous findings show that the dual effects

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 VN1 and VN2 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., 1983). 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 Szolcsányi, 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 (CGRP1 and CGRP2) 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 CGRP18–37), 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 CaCl2 and 2% BSA (fraction V) as an essential oncoytic agent. All perfusions were conducted at 25°C, and the perfusate was continuously gassed with carbogen (95% O2/5% CO2) to ensure a constant arterial PO2. 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 VO2 has been described previously (Colquhoun et al., 1988). Values for VO2 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 Sigmacoat before infusion to prevent peptide adhesion to glass surfaces.

In perfusions wherein CP-99994, SR 48968 or CGRP18–37 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 VO2 and PP, we infused CP-99994, SR 48968 or CGRP18–37 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 VO2 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 6–37 were purchased from Auspep (Australia); capsaicin, Sigmactone 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-(3,4 dichlorophenyl) 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 VN1 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.

**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 6–37.** Infusion of the CGRP antagonist CGRP 6–37 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 6–37 , 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 6–37 (ΔVO_2 control, −1.13 ± 0.29 μmol g^{-1} h^{-1}; CGRP 6–37 , −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
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 VO₂ or PP. The co-infusion of increasing doses of SP with phosphoramidon produced a concentration-dependent increase in VO₂ (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 VO₂ increase, whereas the effect on PP plateaued. NKA, also co-infused with phosphoramidon, produced similar effects on hindlimb VO₂ and PP but was approximately 10-fold more potent than SP (fig. 4; table 1). The infusion of NKB, with phosphoramidon, stimu-
lated a small but reproducible change in VO₂; however, maximum VO₂ 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 VO₂ nor vascular tension.

**Effects of capsaicin pretreatment.** Figure 5 shows VO₂ and PP responses to capsaicin in hindlimbs perfused 1, 7 and 14 days after vehicle or systemic capsaicin pretreatment. The stimulation of VO₂ induced by submicromolar concentrations of capsaicin was significantly inhibited 1 day after capsaicin pretreatment (maximum ΔVO₂; control, 0.98 ± 0.23 μmol g⁻¹ h⁻¹; capsaicin-pretreated, 0.08 ± 0.04 μmol g⁻¹ h⁻¹, P < 0.05; 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 < 0.05; fig. 5D). Seven and 14 days after capsaicin pretreatment, the stimulation of VO₂ and the vasoconstriction induced by low concentrations of capsaicin was completely restored, whereas the maximum inhibition of VO₂ by 2 μM capsaicin was significantly enhanced compared with vehicle-pretreated controls (ΔVO₂; 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. 5B, 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 days (fig. 5; table 2). In addition, the EC₅₀ for capsaicin, estimated from the PP concentration-response curves, was significantly (P < 0.01) 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 VO₂ 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 VO₂ by studying the effects of capsaicin pretreatment and neuropeptide antagonists.

Stimulation of VO₂ produced by submicromolar concentrations of capsaicin (VN₁ 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 VO₂ produced by micromolar concentrations of capsaicin (VN₂ 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 VO₂ (fig. 4). NKA was at least 10-fold more potent than SP at stimulating VO₂ 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 VO₂ by submicromolar concentrations of capsaicin is partly mediated by the endogenous release of SP and NKA, which then stimulate VO₂ 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-

---

**Table 1**

Maximum change in perfusion pressure (ΔPP) and oxygen consumption (ΔVO₂), and concentration producing 50 percent of maximum response (EC₅₀) for SP, NKA and NKB in the perfused rat hindlimb

<table>
<thead>
<tr>
<th>Neuropeptide</th>
<th>n</th>
<th>ΔPP E₅₀ (mm Hg)</th>
<th>ΔVO₂ E₅₀ (μmol g⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>5</td>
<td>4.23 ± 0.33</td>
<td>269 ± 23</td>
</tr>
<tr>
<td>NKA</td>
<td>4</td>
<td>6.75 ± 1.18</td>
<td>21.2 ± 3.0</td>
</tr>
<tr>
<td>NKB</td>
<td>5</td>
<td>3.40 ± 0.25</td>
<td>71.8 ± 29.2</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.

† EC₅₀ estimated using mean of E_max (ΔVO₂) for SP and NKA.

---

**Table 2**

Maximum change in perfusion pressure (ΔPP) and oxygen consumption (ΔVO₂), and concentration producing 50 percent of maximum response (EC₅₀) for capsaicin in the perfused rat hindlimb, 1, 7 and 14 days after vehicle- or capsaicin-pretreatment

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>n</th>
<th>Days after Pretreatment</th>
<th>ΔPP E₅₀ (mm Hg)</th>
<th>ΔVO₂ E₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>5</td>
<td>1</td>
<td>30.2 ± 2.2</td>
<td>1.07 ± 0.04</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>5</td>
<td>1</td>
<td>35.8 ± 3.3</td>
<td>0.99 ± 0.07</td>
</tr>
<tr>
<td>Vehicle</td>
<td>4</td>
<td>7</td>
<td>31.0 ± 1.8</td>
<td>1.02 ± 0.05</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>4</td>
<td>7</td>
<td>42.3 ± 0.3</td>
<td>0.74 ± 0.04**</td>
</tr>
<tr>
<td>Vehicle</td>
<td>5</td>
<td>14</td>
<td>29.3 ± 1.8</td>
<td>0.96 ± 0.05</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>5</td>
<td>14</td>
<td>47.0 ± 4.4</td>
<td>0.66 ± 0.05**</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. **P < 0.01 (Student’s t test) from corresponding vehicle pretreated.

---

**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 < 0.05 from control. Values are mean ± S.E.M. of 4 to 6 experiments.
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 (Santioli 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 A$\delta$-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 $\mu$M 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-pre-treated 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 vasodilation 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 VO₂ 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 VO₂ 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 VO₂ 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 VO₂ (VN₁ 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 VO₂ cannot be ruled out, because in the present study, NK1 and NK2 receptor antagonists decreased capsaicin-induced stimulation of VO₂ 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 VO₂ 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 VO₂ 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 VO₂ inhibition.

The results of the present study imply that capsaicin, when infused into the perfused rat hindlimb, stimulates higher-affinity vanilloid receptors (VN₁) that release thermogenic (VO₂-stimulating) peptides. These receptors appear to be neuronal (primary afferent C fiber), given that systemic capsaicin pretreatment ablates the acute VO₂ stimulation response to infused capsaicin. The stimulation of VO₂ 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 VO₂ by releasing endogenous tachykinins that interact primarily with NK2 receptors. Conversely, CGRP had no detectable effect on VO₂ or pressure, which may be due to the use of an almost fully dilated preparation. Indeed, the CGRP antagonist CGRP₁₈₋₃₇, enhanced capsaicin-induced vasoconstriction and inhibition of VO₂, 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


Send reprint requests to: D.P. Geraghty, Department of Biomedical Science, University of Tasmania, Launceston, Australia 7250.