Evidence for Functional Responses to Sensory Nerve Stimulation of Rat Small Mesenteric Veins

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ABSTRACT
Sensory C-fibers have been implicated in the control of vascular tone and are believed to be predominantly arteriolar in the microvasculature. There have been no direct investigations into the effects of C-fiber activation in venous microvessels. Therefore, we have investigated the effects of neuropeptides and activation of sensory C-fibers in rat small mesenteric veins. Small second- or third-order veins were dissected from the rat mesentery and mounted in a tension myograph for measurement of reactivity. Neither substance P or calcitonin gene-related peptide (CGRP) relaxed precontracted veins. However, substance P caused a concentration-dependent contraction. The curve was shifted to the right in a concentration-dependent manner by the tachykinin receptor antagonist RP 67,580 (0.1–1 μM). To activate sensory C-fibers, capsaicin was applied. Capsaicin had no contractile activity in these vessels but caused concentration-dependent relaxation. This response was significantly attenuated in veins taken from animals in which C-fibers had been largely destroyed (P < .001, n = 5) and in vessels that had been pretreated with the vanilloid receptor blocker ruthenium red (P < .01, n = 5). Endothelial denudation (n = 6) also abolished the response, but the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (100 μM, n = 5) did not inhibit the response; Nω-nitro-L-arginine methyl ester (100–300 μM, n = 4) did inhibit the response. The guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one also significantly attenuated the response (n = 5). The cyclooxygenase inhibitor indomethacin (5 μM, n = 5) and the CGRP receptor antagonist CGRP8–37 (1 μM) were without effect. These results demonstrate that capsaicin, a selective C-fiber activator, relaxes small veins in an endothelium-dependent but CGRP- and substance P-independent manner, and they demonstrate that the venous side of the microcirculation responds directly to sensory stimulation.

Sensory nerves are involved in the control of vascular tone and appear to play an important role in certain types of vascular inflammation. The proinflammatory nature of neuropeptides released from sensory C-fibers has been demonstrated in several models of inflammation, and neurogenic components have been identified in inflammatory disease states, including rheumatoid arthritis (Levine et al., 1985) and asthma (Barnes, 1986).

Activation of sensory C-fibers may be achieved in several ways, including application of the agent capsaicin, the active ingredient of ‘chili pepper’ (Jancso et al., 1967), which selectively activates sensory C-fibers (for reviews, see Holzer, 1991; Dray, 1992), or direct electrical stimulation of nerve fibers. C-fibers are located within the arteriolar side of the microcirculation (e.g., Fleming et al., 1989; Baluk et al., 1992), and activation of these nerves results in the release of the neuropeptides CGRP and SP (Holzer, 1991). It is generally assumed that C-fibers do not innervate the venous side of the microcirculation (e.g., McDonald, 1988; Baluk et al., 1992). Indeed, although there are studies demonstrating the innervation of larger veins, such as human saphenous vein (Herbst et al., 1992), there is no immunohistochemical evidence demonstrating SP or CGRP immunoreactivity in the walls of small veins. When arteriolar C-fibers are activated, CGRP is thought to act at the arteriolar level to produce vasodilatation, whereas SP travels downstream to increase venular permeability. Together, these effects produce greater net edema than does either alone. This synergism has been attributed to an increase in the hydrostatic pressure (due to selective arteriolar dilatation), coupled with increased movement of fluid out of the permeable venules.

Synergism between CGRP and SP has been demonstrated in the skin of rabbits and rats (Brain and Williams, 1985; Gamse and Saria, 1985). However, this has not been a universal finding, and other studies have demonstrated that CGRP has an inhibitory effect (Raud et al., 1991) or no effect (Ekblom et al., 1993) on the response to agents that increase venular permeability. These studies have all relied upon exploring the effects of the neuropeptides in vivo or in intact

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ABBREVIATIONS: CGRP, calcitonin gene-related peptide; L-NAME, Nω-nitro-L-arginine methyl ester; NK, neuropeptide; L-NMMA, Nω-nitro-L-arginine methyl ester; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; SP, substance P.
perfused vascular beds in vitro. In these integrated experimental systems, it is difficult to distinguish between direct effects of neurogenic stimulation on the component parts of the vasculature. To overcome this problem, we have undertaken studies of isolated microvessels in vitro. We previously demonstrated that C-fiber activation in small arteries relaxes constricted vessels and inhibits the contractile responses to sympathetic stimulation (Ahluwalia and Vallance, 1996a). We have now investigated the effects of neuropeptides and capsaicin on the reactivity of isolated small veins of the microcirculation of the rat mesentery. These results have been presented in preliminary form to The British Pharmacological Society (Ahluwalia and Vallance, 1996b).

Methods

Preparation of vessels. Male Wistar rats (240–280 g) were stunned and the neck dislocated. The mesentery was removed and placed in oxygenated cold (4°C) Krebs solution (composition, in mM: NaCl, 118; KCl, 4.69; CaCl₂, 2.5; KH₂PO₄, 1.18; MgSO₄, 1.18; NaHCO₃, 25; glucose, 11). Small second- or third-order veins were dissected out and mounted in cold Krebs‘ solution as ring preparations suspended between two stainless steel wires of 40-μm diameter, in an automated myograph (JP Trading, Aarhus, Denmark), for the measurement of isometric tension (Mulvany and Halpern, 1977). After vessels were mounted, the temperature of the bath was raised to 37°C and the Krebs solution was bubbled with 5% CO₂ in O₂ (pH 7.4). After a 45-min equilibration period, vessels were stretched in a stepwise manner to determine the relationship between the passive tension and the internal circumference, according to Laplace’s equation. They were then stretched to 90% of the diameter achieved when the vessel was under a transmural pressure of 20 mm Hg (i.e., the pressure approximating conditions in vivo). Vessels were repeatedly constricted with the thromboxane A₂-mimetic U-46619 (0.1 μM), until the response was constant, before experimentation. Integrity of the endothelium was determined by assessing the relaxation response to bradykinin (1 μM) in vessels submaximally precontracted with U-46619 (10–100 nM). In experiments where removal of the endothelium was required, a hair was passed through the lumen of the vessels before measurement of the bradykinin response. Endothelium was considered removed when responses to bradykinin were <10% relaxation.

Effects of exogenous neuropeptides. The effects of CGRP (0.1–10 nM) and SP (1–10,000 nM) were determined under conditions of basal tone and in preparations in which stable tone had been induced with U-46619 (10–100 nM). In precontracted vessels, contractile concentration-response curves for SP were repeated until constant, with a 45-min washing period between each curve. When the curves were constant, tissues were incubated with the NK1 receptor antagonist RP 67,580 (0.1–1 μM, 30-min pretreatment), the inactive enantiomer RP 68,651 (1 μM) or the NK2 receptor blocker SR 48968 (1 μM, 2-hr pretreatment). At the end of each experiment the maximal contraction in response to U-46619 (1 μM) was determined.

Responses to capsaicin. Effects of capsaicin were determined under conditions of basal tone and in preparations in which tone had been induced with U-46619 (10–100 nM). In precontracted preparations, a single relaxation concentration-response curve for capsaicin (1–30 μM) was constructed. In some animals C-fibers were removed chemically before experimentation, by systemic treatment with capsaicin (50 mg/kg) or by s.c. administration of capsaicin once 4 days before use. Veins from these animals (or vehicle-treated controls) were removed, and the response to capsaicin was measured. This systemic treatment with capsaicin results in approximately 90% selective destruction of C-fibers in adult rats (Game, 1982; Maggi et al., 1988). To determine whether capsaicin was acting through activation of the vanilloid receptor, vessels were pretreated with ruthenium red (30 μM) for 30 min (Dray et al., 1990; Maggi et al., 1993) before construction of the concentration-response curve for capsaicin. To determine whether the effect of capsaicin was due to activation of CGRP receptors, the effect of the CGRP receptor antagonist CGRP₉⁻₁₇ (1 μM) was investigated. The antagonist was incubated with the vessels for 15 min before construction of the capsaicin concentration-response curve.

Results

The mean diameter of veins used in the present study was 230 ± 13.2 μm (n = 48).

Effects of exogenous neuropeptides. SP (1–1000 nM) caused a concentration-dependent contraction of precontracted veins (see fig. 1 for typical contractile response curve). The concentration-response curve was shifted to the right, in a concentration-dependent manner, by the NK₁ receptor antagonist RP 67,580 (0.1, 0.3 and 1 μM, n = 5 for each concentration), to give concentration ratios of 1.6 ± 0.3 and 3.0 ± 0.7 with 0.1 and 0.3 μM, respectively; with 1 μM a maximum was not reached in all experiments, but a range of 5.0 to >100 μM was achieved. The EC₅₀ for SP in these studies was 1.1 ± 0.2 μM (n = 15). The inactive enantiomer RP 68,651 (1 μM) had no effect on the response to SP (n = 4, not significant, data not shown), nor did NK₂ receptor blockade with SR 48,968 (1 μM, n = 4, data not shown). CGRP had...
no effect in unprecontracted veins, and neither CGRP (0.1–10 nM, \( n = 3 \)) nor SP (0.1–1 \( \mu \text{M} \), \( n = 3 \)) had any relaxant effects on vessels submaximally precontracted with U-46619 (10–100 nM). Pretreatment with CGRP (10 nM) did not alter the response to SP (1–1000 nM, \( n = 3 \)).

**Effects of capsaicin.** Capsaicin had no effect on unprecontracted vessels but caused a concentration-dependent relaxation of precontracted vessels (fig. 2). The responses to capsaicin were significantly (\( P < .001 \)) attenuated in animals denervated of C-fibers by pretreatment with capsaicin (\( n = 5 \)) (fig. 3), compared with the responses in animals that had been treated with vehicle only (\( n = 5 \)). The responses to bradykinin (to check for intact endothelium) were not significantly different in the two groups.

Responses to capsaicin were significantly attenuated by ruthenium red (30 \( \mu \text{M} \), \( n = 5 \)), which caused a 10-fold shift to the right of the concentration-response curve (fig. 4), whereas CGRP\(_{8-37}\) had no effect on the responses to capsaicin (\( n = 3 \), data not shown). Indomethacin (5 \( \mu \text{M} \), \( n = 5 \)) had no significant effect (fig. 4).

L-NMMA (100 \( \mu \text{M} \)) had no effect; l-NAME (100 \( \mu \text{M} \)) appeared to attenuate the responses to capsaicin, but this did not reach significance. However, using the higher concentration of 300 \( \mu \text{M} \), l-NAME caused significant inhibition of the responses to capsaicin (fig. 5A). Guanylate cyclase inhibition, using ODQ, significantly inhibited the responses to capsaicin. The inhibitory effects of this concentration were demonstrated by the fact that the response to glyceryl trinitrate (2 \( \mu \text{M} \)) of 37 ± 3 (\( n = 4 \)) was completely abolished in the presence of ODQ. Endothelial denudation abolished the relaxation response to capsaicin (\( n = 6 \)) (fig. 5B).

**Discussion**

This study demonstrates that local activation of C-fibers with capsaicin relaxes small mesenteric veins. This effect was endothelium-dependent but was not mediated by SP or CGRP and did not involve generation of prostanoid but did appear to involve NO. These results are the first direct dem-
The finding that small veins respond to capsaicin in the same way as do small arteries (Ahluwalia and Vallance, 1996a) suggests that both vessel types possess functionally active sensory C-fibers that respond to the same stimuli.

CGRP is released upon activation of arterial C-fibers and appears in the venous effluent of perfused beds stimulated by capsaicin or electrical field stimulation (Manzini et al., 1991). However, in the vessels we studied, CGRP had no effect on venous tone and therefore is unlikely to contribute to the capsaicin response. Our finding in isolated veins is similar to results from studies in the perfused mesentery, demonstrating that CGRP has no effect on the reactivity of the venous side of the mesenteric microcirculation (Claing et al., 1992). Similarly, studies in humans have demonstrated that CGRP does not dilate capacitance vessels of the hand (Marshall et al., 1988) or human saphenous vein (McEwan et al., 1988). In contrast, a recent study using intravital microscopy of the rat cremaster muscle demonstrated that capsaicin, applied topically, dilates arterioles and venules (Kim et al., 1995) and that the response is blocked by the CGRP receptor antagonist CGRP 8–37. The differences between our study and this earlier study might be due to differences between vascular beds. However, it is possible that the capsaicin-induced and CGRP-dependent venodilatation seen in the in situ cremaster muscle preparation is due to an effect of a mediator released from the arterial side of the circulation in response to CGRP, rather than a direct effect of CGRP on the veins themselves.

The relaxant effects of capsaicin we observed are unlikely to be due to SP. SP had no effect on the precontracted preparations but caused reproducible concentration-dependent contractions in uncontracted veins. This response was mediated by the activation of NK1 receptors, because the selective nonpeptide NK1 receptor antagonist RP 67,580 antagonized the responses, whereas the inactive enantiomer RP 68,651 had no effect. The apparent efficacy of RP 67580 is somewhat lower than would be expected for the rat NK1 receptor, normally with IC50 values in the 1 nM range (for review, see Maggi et al., 1993). The differences in the present study may reflect the involvement of other tachykinin receptors in mediation of this response, but the lack of effect of SR48968 excludes a significant NK2 component. The EC50 for SP is also relatively high for a pure NK1 receptor response, indicating that SP may not be the natural agonist for this system in this preparation. Interestingly, it was demonstrated in the perfused rat mesentery that the vasoconstriction produced after electrical stimulation was attenuated by NK1 receptor antagonism, despite the insensitivity of this preparation to CGRP (Claing et al., 1992). The use of selective NK1 and NK3 receptor agonists such as septide and senktide would help to determine the exact tachykinin receptor subtypes involved in mediation of the responses seen in this preparation. Furthermore, the recent development of NK3 receptor antagonists may also help to clarify the receptor systems involved.

In contrast to the contractile effect of SP, capsaicin did not cause constriction in unpreconstricted vessels; when vessels were preconstricted to an approximately EC50 level with U-46619, capsaicin relaxed the veins. These observations suggest that, under these experimental conditions, SP was not released in quantities sufficient to alter venous tone. Our functional study demonstrating that neither CGRP nor SP is likely to be involved in the relaxant capsaicin response is consistent with anatomic studies that failed to detect CGRP or SP-like immunoreactivity surrounding small veins. Whereas the anatomic studies have been interpreted as sug-

![Fig. 5. Role of endothelial NO in capsaicin-induced relaxation of rat small veins. A, concentration-response curves for capsaicin in the absence (n = 11) (●) and presence of l-NMMA (100 μM, n = 5) (▲), l-NAME (100 μM, n = 6) (△), l-NAME (300 μM, n = 3) (◆) and ODQ (1 μM, n = 4) (○). B, concentration-response curves for capsaicin constructed in preparations with intact endothelium (n = 9) (●) and endothelium-denuded preparations (n = 5) (◆). Values shown are mean ± S.E.M. *P < .05; **P < .001.](image-url)
gesting that there is no sensory innervation of the venous microcirculation, our study clearly demonstrates that the nerves do respond to local sensory stimulation. Consistent with previous studies (Kim et al., 1995), removal of the endothelium abolished the relaxant response to capsaicin, but indomethacin given in doses sufficient to inhibit cyclooxygenase had no effect. The use of NO synthase inhibitors in this system produced varied responses. L-NMMA (100 µM) had no significant effect on the responses to capsaicin, whereas L-NAME (100 and 300 µM) produced a concentration-dependent rightward shift of the capsaicin response, suggesting that NO is involved. The apparently increased efficacy of L-NAME over L-NMMA is not surprising, because it is known that L-NAME is more potent at inhibiting NO synthase activity than does L-NAME (e.g., Salter et al., 1995). Confirmation of involvement of some NO was obtained with the use of the specific guanylate cyclase inhibitor ODQ (Garthwaite et al., 1995). The concentration of ODQ used in these studies was previously shown to inhibit guanylate cyclase; in this study, confirmation of this effect was obtained because the relaxation response to the NO donor glyceryl trinitrate was completely blocked in the presence of ODQ. Our findings are most consistent with the release of a factor from sensory C-fibers that traverses the thin wall of the vein to stimulate the endothelium to release NO or an NO-containing molecule and cause relaxation of vascular smooth muscle by activating guanylate cyclase. An alternative explanation would be that the endothelium responds to noxious stimuli and that capsaicin might activate the endothelium through a mechanism similar to that by which it activates sensory nerves. Although there is evidence for the presence of sensory neuropeptides within the endothelium (Milner et al., 1989), the vanilloid capsaicin receptor has not been found on cells other than neuronal cells, and studies exploring the effects of physical or chemical denervation show complete loss of capsaicin binding in peripheral tissues of rats (Szallasi et al., 1993), suggesting that neurons are the sole target for capsaicin.

These studies demonstrate for the first time that capsaicin, an agent known to selectively activate sensory C-fibers, relaxes isolated small veins of rats. The response is endothelium-dependent and appears to be mediated by NO. It is independent of prostanoid generation and is not mediated by CGRP or SP. The existence of a dilator sensory system on the venous side of the microcirculation that responds to the same stimuli as the nerves present on the arterial side suggests that in some situations hydrostatic pressure might not be altered in response to sensory nerve activation. These studies demonstrate the importance of examining the reactivity of both sides of the microcirculation individually, and they provide insight into the mechanisms of the diverse (pro- or anti-inflammatory) effects of activation of C-fibers seen in vivo.

References

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