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CGRP and nitric oxide of neuronal origin and their involvement in neurogenic vasodilatation in rat skin microvasculature

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1 Sensory nerves are important for the initiation of neurogenic inflammation and tissue repair. Both calcitonin gene-related peptide (CGRP) and nitric oxide (NO) have been implicated in neurogenic vasodilatation and inflammatory responses.

2 A blister model in the rat hind footpad was used as a site to induce neurogenic vasodilatation in response to antidromic electrical stimulation of the sciatic nerve. Blood flux was monitored with a laser Doppler flow monitor.

3 The quantitative contributions of CGRP and NO to vasodilatation were examined by use of the CGRP receptor antagonist $CGRP_{8-37}$ and NO synthase inhibitors 7-nitroindazole (7-NI), 3-bromo 7-NI and N^G-nitro L-arginine methyl ester (L-NAME). The potential modulatory role of endothelin was examined by use of the ET_A receptor antagonist BQ-123.

4 $CGRP_{8-37}$ (10 μM) was perfused over the blister base before nerve stimulation and continuously throughout the post-stimulation period, resulting in a significant reduction (41%) in the blood flux vascular response.

5 Pretreatment with the specific neuronal NO synthase inhibitors, 7-NI and 3-bromo 7-NI (10 mg kg^{-1}, i.v.), and of the non-specific L-NAME (100 μM), resulted in significant inhibition of the blood flux response (36%, 72% and 57% decrease, respectively). In contrast, 7-NI treatment in young rats pretreated with capsaicin had no further effect on the vascular response, suggesting that the source of NO is the sensory nerves.

6 BQ-123 (10 μM) significantly enhanced the stimulation-induced blood flux response (61% increase). When 7-NI was co-administered with either $CGRP_{8-37}$ or BQ-123, the drug actions were additive, suggesting that there was no interaction between NO and CGRP or endothelin.

7 These data suggest that both NO and CGRP participate in neurogenic vasodilatation in rat skin microvasculature and that this response is modulated by endogenous endothelin.

Keywords: Sensory nerves; CGRP; NO; endothelin; vasodilatation; microvasculature

Introduction

Activation of nociceptive sensory nerves in the skin does not only cause pain (via impulses to the brain) but also elicits signs of inflammation. This neurogenic inflammation is initiated by an axon-reflex in which terminals of primary sensory afferents from neuroeffector junctions with target cells in blood vessels. As a result of the axon reflex, peripheral release of sensory neuropeptides (such as substance P and calcitonin gene-related peptide, CGRP) initiates a cascade of events causing extravasation of plasma and vasodilatation within the local microvasculature (for reveiw see Holzer, 1988). CGRP in particular has been implicated as the major mediator of neurogenic vasodilatation (Delay-Goyet *et al*., 1992; Brain *et al*., 1993).

Neurogenic vascular responses to antidromic electrical stimulation of the saphenous and sciatic nerves have been used extensively as measures of the axon-reflex phenomenon (Lembeck & Holzer, 1979; White & Helme, 1985; Brain *et al*., 1993; Khalil *et al*., 1994). Nitric oxide (NO) has also been implicated in neurogenic inflammation since it is released from mast cells and endothelium, and has been shown to have a role in the vasodilator and inflammatory response to bradykinin (Khalil & Helme, 1992), substance P and vasoactive intestinal polypeptide (Ralevic *et al*., 1995), but not that elicited by CGRP (Grace *et al*., 1987; Ralevic *et al*., 1992).

NO has a well established role in the maintenance of low vascular resistance (Rees *et al*., 1990) and it plays a significant role in neurotransmission in the central and peripheral nervous system (Garthwaite *et al*., 1988; Bredt *et al*., 1990; Bredt & Synder, 1992; Morris *et al*., 1992; Meller & Gebhart, 1993). Histochemical studies have provided evidence for the existence of NO synthase (NOS) in nerve fibres of the dorsal root ganglia (Morris *et al*., 1992), which are known to produce and store sensory neuropeptides, and in human and murine skin (Dippel *et al*., 1993; Goldsmith *et al*., 1993). These observations raise the possibility that NO may be involved in neurogenic vascular responses at the level of the peripheral microcirculation. In addition, it has been suggested that NO may regulate release of CGRP and other neurotransmitters from afferent nerve fibres (Holzer & Jocič, 1994; Hughes & Brain, 1994; Kajekar *et al*., 1995).

The endothelium-derived vasoconstrictor endothelin (ET) may modulate neurogenic plasma extravasation (Brändli *et al*., 1995). There is increasing evidence to suggest complex interactions between endothelin and NO: endothelin stimulates the release of NO while NO inhibits the synthesis of endothelin (Boulanger & Lüscher, 1990; Levin, 1996). Furthermore, acute NO blockade not only potentiates the vasoconstrictor actions of endothelin (Filep *et al*., 1993) but also enhances the synthesis and release of endothelin. Both ET_A and ET_B receptors have been described in rat dorsal skin (Lawrence *et al*., 1995). In addition, endothelin-like immunoreactivity has

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been found in afferent cell bodies (Franco-Cereceda *et al*., 1991), suggesting that endothelin may be produced by neuronal cells. Hence, the interaction between NO and endothelin may have important implications for the neurogenic inflammatory response.

Using inhibitors for neuronal and endothelial NOS (7-nitroindazole, its bromo-derivative and N^G-nitro L-arginine methyl ester (L-NAME) and antagonists of CGRP and endothelin receptors ($CGRP_{8-37}$ and BQ-123, respectively), we set out to determine the relative contributions, and possible interactions of these mediators and modulators, to the vascular response obtained in response to antidromic stimulation of sensory nerves supplying the microvasculature in rat skin.

Methods

Outbred male Sprague-Dawley rats 3 months of age were used. Anaesthesia was induced with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and maintained by supplementary injections. Mean arterial blood pressure was monitored with a COBE pressure transducer attached via a catheter to the right carotid artery. The left jugular vein was cannulated for intravenous administration of the inhibitors or vehicle solutions. Body temperature was maintained at 37°C. Animals were killed by barbiturate overdose at the completion of experiments.

Neonatal capsaicin pretreatment

Neonatal rats were pretreated on the second day of life with a single subcutaneous injection of 50 mg kg^{-1} capsaicin. This treatment has been shown to destroy permanently the majority of sensory nerve fibres (Lembeck & Holzer, 1979) allowing the assessment of the role of sensory fibres in these vascular responses. Experiments were performed at the age of three months. Efficacy of this treatment was confirmed by applying a drop of capsaicin (0.1%) to the eye and recording the subsequent number of eyewipes. Rats were considered capsaicin-denervated if the number of eyewipes was less than 25% of controls.

Blister induction and antidromic stimulation of sensory nerves

A blister was induced on the hindpaw of the anaesthetized rat by applying a vacuum pressure of −40 kPa. This induction period lasted 30 min in both control and capsaicin-pretreated rats. The sciatic nerve at the mid-thigh region was then carefully exposed and cut, the distal portion placed over platinum electrodes and immersed in a warm oil pool formed from the skin flaps of the wound. The electrodes were fixed in such a position that electrical leakage to adjacent nerve and tissue structures was minimized. The surface epidermis of the blister was then removed and a perspex chamber (with inlet and outlet ports) secured over the blister base. Ringer solution and/or receptor antagonists were perfused over the blister surface continuously during the experiment and maintained at 4 ml h^{-1}, as in previous experiments (Ralevic *et al*., 1992; Khalil *et al*., 1996). Activation of the sensory fibres was achieved with a Grass stimulator at 20 V, 5 Hz, 2 ms for 1 min duration. These parameters have been previously used to stimulate efferent C-fibre responses (Lembeck & Holzer, 1979; White & Helme, 1985), to evoke an immediate increase in local blood flow. The time elapsed from the start of the blister induction until sciatic nerve stimulation did not exceed 60 min.

Measurement of cutaneous blood flow

A laser Doppler flowmeter probe was positioned vertically over the exposed blister in the hindpaw via the perspex chamber. The flux output of the laser Doppler monitor is a function of the concentration and the velocity of the red blood cells moving in the tissue penetrated by the laser light. The changes in relative blood flow (as determined by changes in red cell flux) following electrical stimulation of the cut sciatic nerve were continuously displayed on a chart recorder. Raw data were evaluated by calculating the area under the stimulation-evoked response curve (AUC, cm^2) for a post-stimulation period of 20 min. All measurements were made relative to a stable baseline obtained before nerve stimulation. Results are expressed as a percentage of the area (in cm^2) of the response obtained in the appropriate control group of rats. Sodium nitroprusside (SNP, 100 μM), a directly acting smooth muscle vasodilator, was perfused in all rats for 10 min after baselines were re-established following the stimulation period. The baselines did not differ between control and capsaicin-pretreated rats or control and any other acute treatment groups before electrical stimulation.

Administration of NO synthase inhibitors and receptor antagonists

7-Nitroindazole (7-NI) and 3-bromo-7-nitroindazole (3Br-7NI), specific inhibitors of the neuronal isoform of NOS, were injected intravenously (10 mg kg^{-1}) 5 min before nerve stimulation. These drugs were dissolved in absolute ethanol and hot sodium bicarbonate solution (0.5% w/v) at a ratio of 1 : 9. N^G-nitro L-arginine methyl ester (L-NAME, a non-specific NOS inhibitor), BQ-123 (cyclo-D-Trp-D-Asp(ONa)-Pro-D-Val-Len; an antagonist of endothelin receptor type A) and the CGRP receptor antagonist $CGRP_{8-37}$ dissolved in Ringer solution were perfused over the blister base 5 min before and continuously during the stimulation and post-stimulation periods.

Analyses of blood flux response

In each experiment, blood flux responses were measured and normalized by dividing the stimulation-induced area by ratio of the SNP response divided by the mean nitroprusside response for that group of rats. This correction effectively uses the SNP response as an internal standard for individual variations in the blood flux response to stimulation in each preparation.

Statistical evaluation of the blood flux responses was performed by means of independent Student's *t* test. Blood pressure was analysed by means of independent and paired samples *t* test.

Results

Effect of CGRP antagonist on the vascular response to sensory nerve stimulation

Control stimulations resulted in an immediate increase in the blood flux response, with an AUC of 18.9 ± 1.9 cm^2. The contribution of CGRP to this antidromic response was examined by perfusing the CGRP receptor antagonist $CGRP_{8-37}$ over the blister base before sciatic nerve stimulation at several concentrations. This did not alter the baseline blood flux, but concentrations of 1 μM and higher resulted in

significant decreases in the subsequent blood flux response to nerve stimulation (1 μM $-41\pm10\%$ decrease, 10 μM $-77\pm5\%$ decrease, Figure 1). The absolute response to SNP was not altered by any $CGRP_{8\text{-}37}$ concentration.

Effect of NOS inhibitors on the vascular responses

An immediate increase in the blood flux response upon nerve stimulation was observed in this group of control rats receiving vehicle injection, with an AUC of 18.6 ± 1.3 cm^2. Following the administration of the selective neuronal NOS inhibitor, 7-NI (10 mg kg^{-1}, i.v.), blood flux responses to nerve stimulation were significantly attenuated ($36\pm7\%$ decrease compared to control response, Figure 2a). 3-Bromo-7-nitroindazole (3-Br-7-NI, 10 mg kg^{-1}, i.v.) caused a greater inhibition of the stimulation-induced vasodilator responses than that achieved by 7-NI ($72\pm4\%$ decrease, Figure 2a). Neither drug altered systemic arterial blood pressure (Figure 2b) nor baseline blood flux. Unlike these neuronal NOS inhibitors, L-NAME (a non-specific NOS inhibitor) causes hypertension when administered intravenously (Rees *et al*., 1990; Kajekar *et al*., 1995), so in this study L-NAME was perfused locally over the blister base before nerve stimulation. At a concentration of 100 μM (which is submaximal for inhibition of SP-induced responses, Khalil *et al*., 1994), L-NAME also significantly reduced the blood flux response compared to control responses ($57\pm7\%$ decrease, Figure 2a).

Effect of neuronal NOS inhibitor on the vascular response in sensory-denervated rats

Blood flux responses in capsaicin-pretreated rats following electrical stimulation was reduced with an AUC of 5.7 ± 0.6 cm^2 ($30\pm5\%$ decrease compared to control rats (Figure 3a). 7-NI (10 mg kg^{-1}, i.v.) in capsaicin-pretreated rats did not alter the basal or blood flux response induced by nerve stimulation (Figure 3a), nor did it affect mean arterial blood pressure (Figure 3b). Blood pressure in these rats was significantly lower than that of the control rats, but did not drop below 100 mmHg throughout the recording period.

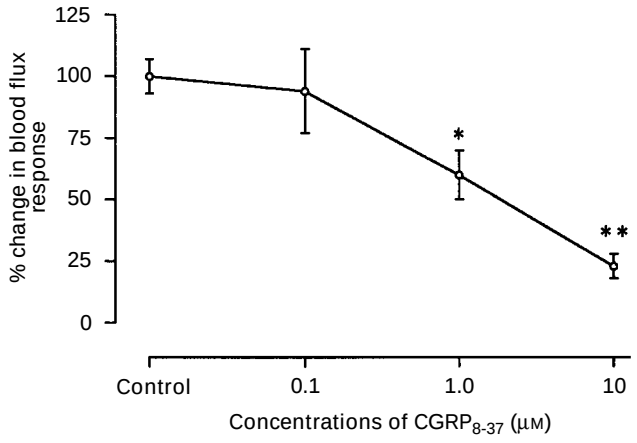

Figure 1 Dose-response curve showing the effect of $CGRP_{8\text{-}37}$ on neurogenic vasodilatation induced by electrical stimulation of the rat sciatic nerve. Results are expressed as percentage change in local skin blood flux in rat hind paw compared with control responses. Concentrations of 1 μM and 10 μM both significantly inhibited the flux response, with 1 μM being a submaximal concentration. Values are mean and vertical lines show s.e.mean. ($n=4-15$). Asterisks denote significant difference from control data ($^*P<0.05$, $^{**}P<0.001$).

Effect of CGRP receptor antagonist combined with 7-NI on the vascular response

Administration of 7-NI or $CGRP_{8\text{-}37}$ before nerve stimulation resulted in significant inhibition of the blood flux responses (36% and 41%, respectively). Simultaneous application of 7-NI with $CGRP_{8\text{-}37}$ resulted in a decrease of $68\pm8\%$ in the blood flux response. The inhibitory effects therefore appear to be additive (Figure 4).

Effect of endothelin receptor antagonist and 7-NI on the vasodilator response

Perfusion of the endothelin receptor antagonist BQ-123 (10 μM) over the blister base for 5 min did not alter the basal blood flux before nerve stimulation. However, it resulted in a significant increase in the blood flux response following stimulation (AUC: 30.4 ± 2.2 cm^2), corresponding to a $61\pm11\%$ increase compared to control data (Figure 5). The

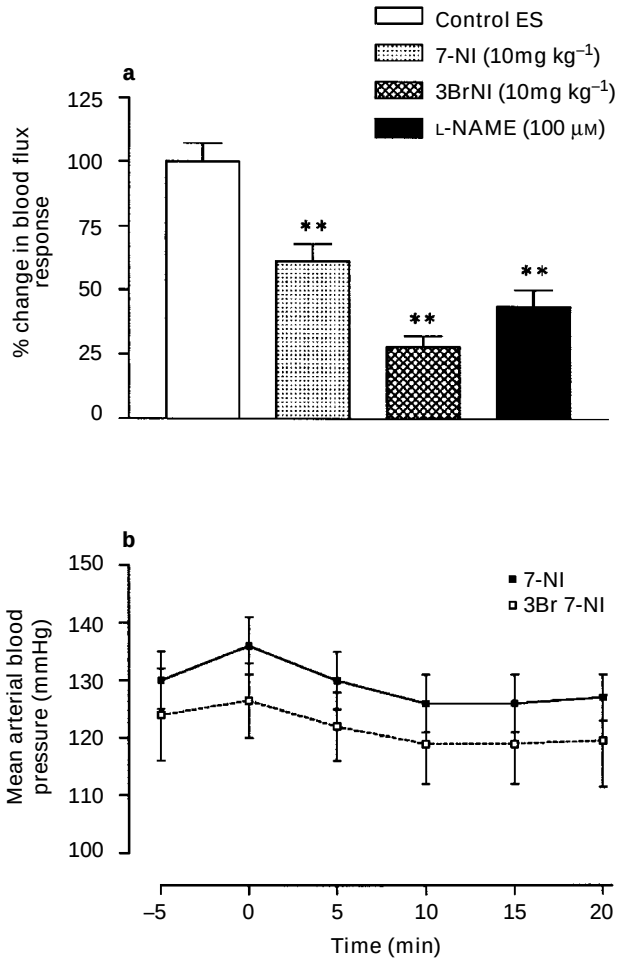

Figure 2 (a) The effect of various NOS inhibitors on blood flux responses induced by electrical stimulation (ES) of the sciatic nerve. 7-NI (10 mg kg^{-1}, i.v.), 3-bromo 7-NI (10 mg kg^{-1}, i.v.) and L-NAME (100 μM – local perfusion) were administered 5 min before and continuously throughout the post-stimulation period. Results are expressed as percentage change in local skin blood flux in rat hind paw compared with control responses. Values are mean $\pm$ s.e.mean. ($n=6-16$). Asterisks denote significant difference from control data ($^{**}P<0.001$). (b) The effect of intravenous 7-NI and 3-bromo 7-NI on mean arterial blood pressure measured before and during the post-stimulation period. Values are mean and vertical lines show s.e.mean. ($n=6$). Sciatic nerve was stimulated at time = 0 min; NOS inhibitors administered at time = -5 min.

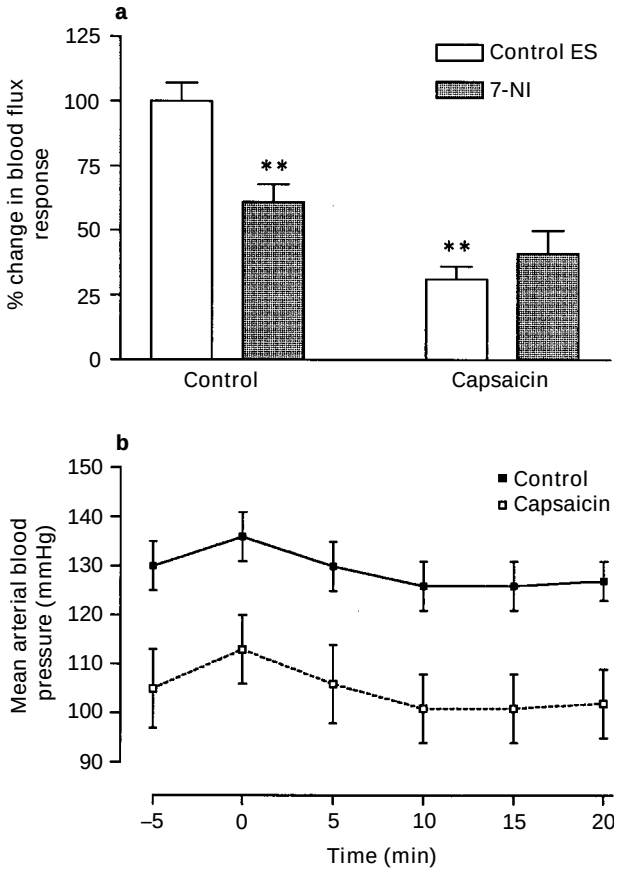

Figure 3 (a) The effect of capsaicin-pretreatment and neuronal NOS inhibition on blood flux responses induced by electrical stimulation (ES) of the sciatic nerve. Results are expressed as percentage change in local skin blood flux in rat hind paw compared with young control responses. 7-NI (10 mg kg^{-1}, i.v.) treatment did not have any significant effect in capsaicin rats (3 months age) Values are mean ± s.e.mean. ($n = 6-16$). Asterisks denote significant difference from control data (**$P < 0.001$). (b) Blood pressures in control and capsaicin-pretreated rats following 7-NI (10 mg kg^{-1}, i.v.) administration. There were no significant changes in blood pressure levels induced by nNOS inhibition in any group of rats. Values are mean and vertical lines show s.e.mean ($n = 5-6$).

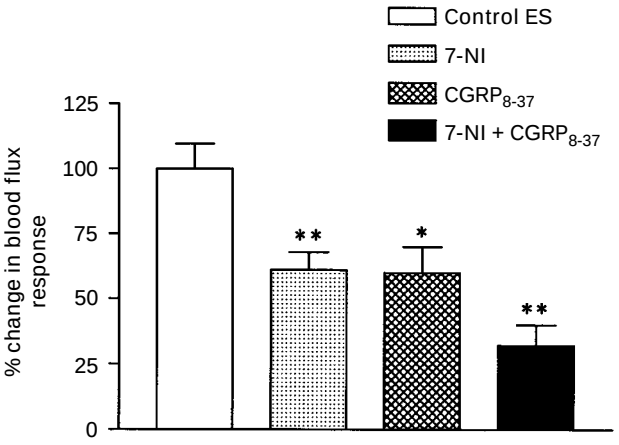

Figure 4 The effect of CGRP receptor antagonist ($CGRP_{8-37}$, 1 μM) and neuronal NOS inhibitor (7-NI, 10 mg kg^{-1}) on the vasodilator responses induced by stimulation of sensory nerves. Results are expressed as percentage change in local skin blood flux in rat hind paw compared with control responses. Both drugs caused a significant inhibition of the subsequent flux response. When $CGRP_{8-37}$ and 7-NI were co-administered, the stimulation-evoked response was further reduced. Values are mean ± s.e.mean ($n = 5-16$). Asterisks denote significant difference from control data (*$P < 0.05$, **$P < 0.001$).

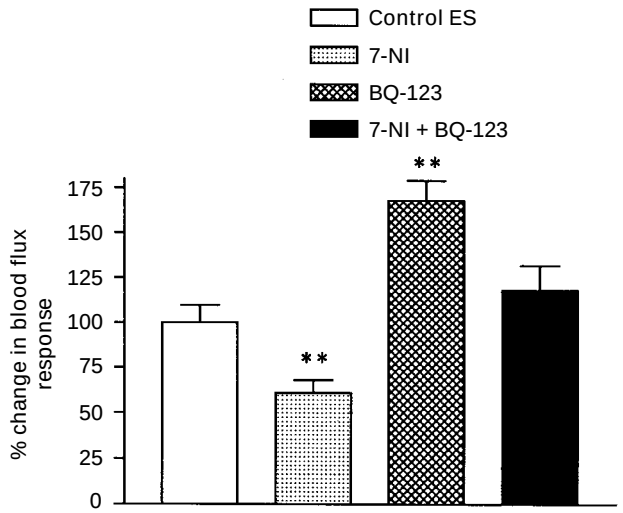

Figure 5 The effect of endothelin receptor antagonist (BQ-123, 10 μM) and neuronal NOS inhibitor (7-NI, 10 mg kg^{-1}) on blood flux responses induced by stimulation of the sciatic nerve. Results are expressed as percentage change in local skin blood flux in rat hind paw compared with control responses. Inhibition of endothelin resulted in a significant increase in the blood flux response. When accompanied by NOS inhibition, the response was not significantly different from control responses. Values are mean ± s.e.mean ($n = 5-16$). Asterisks denote significant difference from control data (**$P < 0.001$).

endothelin antagonist was used at this concentration since it was previously determined in our laboratory to be effective in reversing the constrictor effects of endothelin (Khalil *et al*., 1996). When BQ-123 was applied in conjunction with 7-NI, the resultant vasodilator response was reduced to the level observed in the control rats (Figure 5).

Discussion

The present study showed that both NO and CGRP contribute to the neurogenic vasodilator response obtained to antidromic stimulation of sensory nerves supplying the rat blister base. In this preparation, the source of NO appears to be the nerves, for vasodilatation was blocked by selective inhibitors of neuronal NOS.

Red cell flux as measured by the laser Doppler flow monitor is widely used in studies of localized peripheral blood flow. Since blood pressure did not change in these studies, the integrated response change in flux by time provides a measure of vasodilatation. This has been used to interpret all the blood flux measurements in our study, for neither baseline flux nor blood pressure was altered by any treatments.

The sensory neuropeptides substance P and CGRP are major mediators of neurogenic inflammation. Substance P has a prominent role in plasma extravasation and CGRP is important in the local vasodilator responses (Khalil *et al*., 1988; Delay-Goyet *et al*., 1992; Brain *et al*., 1993; Escott & Brain, 1993). Perfusion of the CGRP receptor antagonist over the blister base before and during nerve stimulation caused a dose-dependent decrease in the blood flux response to nerve stimulation, with 10 μM inducing approximately 77% inhibition. This accords with the previous results of Escott *et al*. (1995), obtained with rat saphenous nerve and trigeminal ganglion stimulation and confirms the role CGRP in neurogenic vasodilatation.

Nitric oxide is an important regulator of microvascular tone with significant roles in the central and peripheral nervous system. In this study, intravenous administration of a specific neuronal NOS inhibitor, 7-NI, led to a significant decrease (36%) in the vasodilator response to nerve stimulation without an affect on systemic arterial blood pressure, as observed by Moore *et al*. (1993). Kajekar *et al*. (1995) found that the oedema formation in rat skin induced by saphenous nerve stimulation was selectively inhibited by 7-NI. These data indicate that NO produced in neural tissues contributes significantly to the neurogenic inflammatory response. Zagvazdin *et al*. (1996) showed that 7-NI also suppressed endothelial NO formation *in vivo*, but a much higher concentration of 7-NI was required for this effect.

3-Bromo 7-nitroindazole was also found to have an inhibitory effect (72%) on the stimulation-induced blood flow which was greater than 7-NI, consistent with the greater potency of 3-bromo 7-NI for rat cerebellar NOS activity *in vitro* (Bland-Ward & Moore, 1995). The inhibition of blood flux response achieved with L-NAME was similar to the inhibition obtained by the specific neuronal NOS inhibitors, suggesting that endothelial NO probably does not play a significant role in sensory antidromic vasodilatation.

Others have recently found that NO is involved in nerve signalling and nociceptive processing in primary afferent fibres (Morris *et al*., 1992; 1994). To investigate further the source of NO responsible for vasodilatation, we examined the effect of 7-NI in a group of rats pretreated as neonates with capsaicin. Neonatal capsaicin-pretreatment induces irreversible destruction of 70–90% of sensory nerves (Lembeck & Holzer, 1979; Nagy *et al*., 1981; Khalil *et al*., 1994). The marked reduction in the response to antidromic nerve stimulation (at C-fibre strength) in these animals reflects sensory nerve fibre destruction. The residual vasodilator response in these animals is most likely mediated by Aδ fibres and the small proportion of capsaicin-insensitive fibres remaining following pretreatment (Janig & Lisney, 1989). Selective inhibition of NOS with 7-NI did not alter the residual vasodilatation in capsaicin-pretreated rats. Hence, capsaicin-sensitive sensory fibres appear to be the source of NO involved in stimulation-induced vasodilatation. The basal blood pressure levels were significantly reduced in the capsaicin-pretreated rats compared to controls. However, it has previously been shown that decreases in blood pressure exceeding those in this study do not affect neurogenic inflammatory responses (Coderre *et al*., 1989).

We and others have previously shown that the vasodilator action of CGRP in the microvasculature is independent of NO (Ralevic *et al*., 1992; Holzer & Jocič, 1994; Hughes & Brain, 1994; Kajekar *et al*., 1995). However, the studies of Brain and colleagues suggested that the actual release of peptide neurotransmitters from sensory nerve fibres is regulated by NO. When the selective neuronal NOS inhibitor 7-NI and the receptor antagonist $CGRP_{8-37}$ were applied simultaneously, we observed a greater reduction in the stimulation-evoked vascular response than that observed with either 7-NI or $CGRP_{8-37}$ alone. This result suggests that in our preparation, both CGRP and NO (of neuronal origin) contribute independently to the vasodilator effect in the microvasculature. The study of different models of cutaneous vascular responses and different stimulation parameters could account for the discrepancy between our results and those of Holzer and Jocič (1994), and Kajekar *et al*. (1995).

The physiological balance between NO and the vasoconstrictor endothelin is complex and regulated in several ways (Boulanger & Lüscher, 1990; Levin, 1996). Imbalances would lead to problems in the control of vascular tone and could be the underlying cause of many cardiovascular diseases. Endothelin does appear to be an endogenous regulator of vascular tone in man (Haynes & Webb, 1994), but in this work and our previous study, the endothelin antagonist did not alter maintenance of resting blood flow (Khalil *et al*., 1996). Furthermore, blockade of ET_A receptors caused a significant increase in the vasodilator response following nerve stimulation, suggesting a role for endogenous endothelin in modulating the vascular response. Blockade of ET_A receptors together with inhibition of neuronal NOS caused 18% increase in the vascular response to nerve stimulation, compared to a 68% increase achieved with BQ-123 alone. Thus 7-NI and BQ-123 together produce an additive effect, suggesting that there is no specific interaction between NO and endothelin other than algebraic summation in terms of neurogenic vasodilatation in the peripheral microcirculation.

In conclusion, these results suggest that both NO and CGRP are involved in neurogenic vasodilatation induced by antidromic stimulation of sensory nerve fibres in rat skin microvasculature. The NO contribution appears to originate from capsaicin-sensitive C-fibres. In addition, endogenous endothelin modulates neurogenic vasodilatation, although we failed to find evidence for additional interactions between NO and CGRP, or NO and endothelin in the control of microvascular tone in this preparation.

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