Nitric oxide and sensory nerves are involved in the vasodilator response to acetylcholine but not calcitonin gene-related peptide in rat skin microvasculature

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1 The contributions of sensory nerves and nitric oxide (NO) to vasodilator responses to acetylcholine (ACh) and calcitonin gene-related peptide (CGRP) were examined in rat skin microvasculature with a laser Doppler flowmeter to monitor relative blood flow.

2 Perfusion of ACh (100 μ M; for 30 min) over a blister base on the rat hind footpad elicited microvascular vasodilatation and this response was not sustained. CGRP (1 μ M; 10 min perfusion) also elicited vasodilatation and this response was maintained even when CGRP was no longer in contact with the blister base.

3 The vasodilator response to ACh was significantly smaller in rats pretreated as neonates with capsaicin to destroy primary sensory afferents than it was in age-matched controls. The vasodilator response to CGRP was unaffected by capsaicin pretreatment.

4 Selective inhibitors of NO synthase, N^G-nitro-L-arginine (L-NOARG) and N^G-monomethyl-L-arginine (L-NMMA) (both at 100 μ M) attenuated the vasodilator response to ACh in control rats, but had no effect on the vasodilator response to CGRP. There was a significant L-NOARG-resistant component in control rats while in capsaicin-treated rats the vasodilator response to ACh was virtually abolished by L-NOARG. The inactive stereoisomer N^G-monomethyl-D-arginine (100 μ M) did not affect the vasodilator response to ACh.

5 The efficacy of L-NOARG and L-NMMA as inhibitors of endothelium-dependent responses was confirmed by use of an endothelium-dependent vasodilator, the calcium ionophore A23187 (100 μ M; 10 min perfusion). Vasodilatation to A23187 was strongly attenuated by both L-NOARG and L-NMMA.

6 These results suggest that sensory nerves and NO are both involved in the dilatation produced by ACh in rat skin microvasculature. A component of the vasodilator response elicited by ACh involves a direct action on the microvascular endothelium with subsequent generation of NO, while an additional component is elicited via activation of sensory nerves. The vasodilator mediator(s) released by ACh from sensory nerves acts largely independently of NO.

7 The vasodilator response to CGRP is independent of a prejunctional action on sensory nerves and of NO.

Keywords: Nitric oxide; endothelium; microvasculature; sensory nerves; acetylcholine; calcitonin gene-related peptide; neurogenic inflammation

Introduction

Neurogenic inflammation constitutes a complex series of events involving the release of peptide neurotransmitters, particularly substance P and calcitonin gene-related peptide (CGRP), from sensory nerve endings in the skin. CGRP is a potent vasodilator in human and animal skin (Brain *et al.*, 1985; Thom *et al.*, 1987). In rat skin, CGRP may potentiate the oedema induced by substance P and other tachykinins, while substance P causes vasodilatation and an increase in vascular permeability and may modulate the vasodilatation induced by CGRP (Brain & Williams, 1985). Neurogenic inflammation additionally involves the release of mediators from mast cells and activation of sensory nerves (Lembeck & Holzer, 1979; Foreman *et al.*, 1983; Barnes *et al.*, 1986). Nitric oxide (NO) acts as a transduction molecule in many biological systems (Moncada & Higgs, 1990) and is also likely to be involved in neurogenic inflammation since it may be released from mast cells (Salvemini *et al.*, 1990) as well as from the endothelium and has been shown to have a role in

the inflammatory response to substance P (Hughes *et al.*, 1990; Ralevic *et al.*, 1991).

Acetylcholine (ACh) causes pain when applied to blister bases in man (Armstrong *et al.*, 1953). It has been suggested that ACh released from postganglionic sympathetic nerve fibres contributes to the inflammatory response since antidromic vasodilatation and neurogenic plasma extravasation are reduced by atropine (Couture *et al.*, 1985; Couture & Kerouac, 1987; Low & Westerman, 1989). In large blood vessels it is well established that vasodilatation due to ACh is largely mediated through an action on the vascular endothelium with subsequent release of endothelium-derived relaxing factor or NO (Furchgott, 1983; Palmer *et al.*, 1988). In cutaneous microvasculature, sensory nerves may also be involved, since ACh has been shown to excite perivascular nociceptive C-fibres in man (Westerman *et al.*, 1987; Parkhouse & LeQuessne, 1988), rabbit (Juan, 1982) and cat (Fjallbrandt & Iggo, 1961).

It was the aim of the present study to examine the contribution of sensory nerves and of NO to ACh- and CGRP-induced inflammatory responses in rat skin microvasculature. The role of sensory nerves was examined in rats pretreated as neonates with capsaicin to destroy selectively these nerves,

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while the involvement of NO was examined with two selective inhibitors of NO synthase, N^G-nitro-L-arginine (L-NOARG) and N^G-monomethyl-L-arginine (L-NMMA) (Palmer *et al.*, 1988; Rees *et al.*, 1989; Dubbin *et al.*, 1990; Moore *et al.*, 1990). Some of these results have been published in preliminary form (Ralevic *et al.*, 1991).

Methods

Sprague-Dawley rats of either sex with an average weight of 200–250 g were used. Anaesthesia was induced with pentobarbitone sodium (60 mg kg⁻¹, i.p.). Further doses of 15 mg kg⁻¹ anaesthetic were administered intermittently to ensure that rats were kept under a constant state of anaesthesia. This method of anaesthesia was previously shown not to alter the basal plasma extravasation or sympathetic tone (Khalil & Helme, 1989). Body temperature of the rats was maintained at 37°C. At the end of the experiment animals were killed by barbiturate overdose.

Blister induction and experimental protocol

A blister was induced in the right hind footpad of the rat by applying a vacuum pressure of -40 kPa for approximately 30 min, with a metal suction cap heated to 40°C. The surface epithelium was removed and the foot of the rat secured in a perspex chamber with inlet and outlet ports. Ringer solution was perfused over the surface of the blister at 4 ml h⁻¹ by means of a peristaltic pump (Microperpex S, LKB, Sweden) (Khalil & Helme, 1989). Relative blood flow was monitored over time with a laser Doppler flowmeter (Periflux, PF2B, Perimed, Sweden) via a probe placed in a central port immediately above the blister base; blood flux (volt; V) was recorded on a chart recorder.

An initial equilibration period of 30 min was allowed, during which time a stable baseline was achieved. In control experiments ACh (100 µM) was perfused for 30 min over the blister base. In experiments with L-NOARG, L-NMMA or the inactive stereoisomer N^G-monomethyl-D-arginine (D-NMMA) (all at 100 µM) these agents were perfused for 14 min before and during ACh perfusion. (The concentration of L-arginine analogues used was predetermined to cause an attenuation of vasodilator responses to endothelium-dependent agents without a significant change in the baseline). CGRP (1 µM) was perfused for 10 min at the end of each experiment (20 min after cessation of ACh perfusion). Concentration-response curves for ACh (10, 100 and 1000 µM) and CGRP (0.1, 1 and 10 µM) were produced by perfusing these agents over the surface of the blister base; because of the effects of desensitization, responses to single concentrations were established in separate animals. In a separate series of experiments A23187 (100 µM) was perfused for 10 min over the blister base. All agonists were used at submaximal concentrations; the time of perfusion used was based on the vasodilator characteristics of these agents. Experiments with L-NOARG were repeated in rats having undergone destruction of primary sensory afferents by neonatal capsaicin treatment (Jancso *et al.*, 1977).

Capsaicin pretreatment

Animals were given a single dose of capsaicin, 50 mg kg⁻¹, subcutaneously, on the fourth day of life. The effectiveness of the neonatal capsaicin treatment was tested prior to each experiment by the eye-wiping response to topical application of capsaicin to the cornea (0.1% capsaicin in alcohol) (Khalil & Helme, 1990).

Drugs

Calcitonin gene-related peptide was obtained from Auspep, Melbourne, Australia. Acetylcholine perchlorate, capsaicin

(8-methyl-N-vanillyl-6-nonenamide), and N^G-nitro-L-arginine were from Sigma Chemical Company, U.S.A. A23187 (free acid) and N^G-monomethyl-L-arginine acetate were from Calbiochem, U.S.A. N^G-monomethyl-D-arginine acetate was from the Institute of Drug Technology (Boronia, Victoria). Capsaicin was dissolved in a saline solution containing 10% Tween 80 and 10% alcohol. A23187 was initially dissolved in dimethylsulphoxide (DMSO) and then diluted in Ringer solution. The appropriate solvent controls were carried out with DMSO. All other drugs were dissolved in Ringer solution.

Statistics and expression of data

Relative blood flow responses were measured as the area under the response curve (cm²) with a digital planimeter (Tamaya, Japan). For ACh and A23187 this was the area during the entire perfusion; for CGRP this was the area during 8 min of perfusion since the response was maintained. Maximum height of responses was also measured. Small non-specific solvent effects were accounted for in experiments using A23187 by performing controls with DMSO at the appropriate concentration. Results were expressed as mean ± s.e.mean. Statistical analyses were performed by analysis of variance with *post hoc* Student-Newman-Keuls pairwise comparisons where necessary. Type 1 error rate was set at $\alpha = 0.05$ for all tests.

Results

Effect of L-NOARG, L-NMMA and D-NMMA on the vasodilator response to ACh

In control rats, ACh (100 µM; perfused for 30 min) produced an initial rapid vasodilator response in the rat skin microvasculature which reached a maximum and then gradually subsided (Figure 1). L-NOARG (100 µM) and L-NMMA (100 µM) but not D-NMMA (100 µM) caused a significant reduction in the area of the vasodilator response to ACh (Figure 2). This inhibition was manifested primarily as a reduction in the time-dependent maintenance of the response; in the presence of L-NOARG or L-NMMA the vasodilator response to ACh returned to baseline (Figure 1). There was no significant difference between the inhibitory effects of L-NOARG or L-NMMA (both at 100 µM) on the area of the vasodilator response to ACh. Only L-NMMA significantly attenuated the maximum height of the vasodilator response to 100 µM ACh (6.98 ± 1.12 ($n = 6$), 4.8 ± 0.36 ($n = 5$) and 2.95 ± 0.64 ($n = 4$) cm in control, L-NOARG- and L-NMMA-

Figure 1 Representative trace showing the effect of N^G-nitro-L-arginine (L-NOARG, 100 µM) on vasodilator responses (blood flux, V) of the rat skin microvasculature to acetylcholine (ACh; 100 µM perfused for 30 min) and calcitonin gene-related peptide (CGRP, 1 µM perfused for 10 min). Unbroken line indicates control responses; broken line indicates responses in the presence of L-NOARG, introduced 14 min prior to and during challenge with ACh and CGRP.

Figure 2 Effect of N^G -nitro-L-arginine (L-NOARG), N^G -monomethyl-L-arginine (L-NMMA) or D-NMMA on the dilatation response of the rat skin microvasculature to acetylcholine (ACh; $100 \mu\text{M}$). Open column = ACh alone; solid column = ACh + L-NOARG ($100 \mu\text{M}$); narrow hatched column = ACh + L-NMMA ($10 \mu\text{M}$); wide hatched column = ACh + L-NMMA ($100 \mu\text{M}$); cross hatched column = ACh + D-NMMA ($100 \mu\text{M}$). Columns represent the means of 4–7 separate experiments with vertical bars indicating s.e.mean. *Indicates significant difference from control response.

treated rats respectively). The maximum height of the vasodilator response to ACh at a concentration of 1 mM was significantly attenuated by L-NOARG (Figure 4). A lower concentration ($10 \mu\text{M}$) of either L-NOARG or L-NMMA (Figure 2) had no significant effect on the vasodilator response to ACh.

Effect of L-NOARG and L-NMMA on the vasodilator response to CGRP

The vasodilator response to CGRP ($1 \mu\text{M}$, 10 min perfusion) was characterized by an initial rapid vasodilatation which reached a maximum and was maintained even after CGRP was removed from the perfusate (Figure 1). Previous studies in our laboratory have shown that this vasodilator response can be maintained for several hours after cessation of perfusion with CGRP. L-NOARG ($100 \mu\text{M}$) or L-NMMA ($100 \mu\text{M}$) had no significant effect on the vasodilator response to CGRP (Figure 3). A concentration-response curve showed that L-NOARG had no effect on CGRP at concentrations of 0.1 , 1 or $10 \mu\text{M}$ (Figure 4).

Effect of L-NOARG and L-NMMA on the vasodilator response to A23187

The vasodilator response to A23187 ($100 \mu\text{M}$) was slow in onset (Figure 5). L-NOARG and L-NMMA (both at $100 \mu\text{M}$) markedly inhibited the area of the response (Figure 6). In all but one of the five animals L-NOARG abolished the vasodilator response due to A23187. L-NOARG, but not L-NMMA significantly inhibited the maximum height of the vasodilator response (2.5 ± 0.41 , 0.36 ± 0.29 and $1.72 \pm 0.62 \text{ cm}$ ($n = 5$) in control, L-NOARG- and L-NMMA-treated rats respectively).

Effect of capsaicin pretreatment on vasodilator responses to ACh and CGRP

In rats pretreated with capsaicin there was a significant reduction in the area of the vasodilator response to ACh (Figure 7). There was also a significant reduction in the maximum height of the response between these groups (6.98 ± 1.12 and $3.97 \pm 0.37 \text{ cm}$ ($n = 6$) in control and capsaicin treated rats respectively). Capsaicin pretreatment had no significant effect on vasodilator responses to CGRP (Figure 8).

Figure 3 Effect of N^G -nitro-L-arginine (L-NOARG), or N^G -monomethyl-L-arginine (L-NMMA) on the dilatation response of the rat skin microvasculature to calcitonin gene-related peptide (CGRP, $1 \mu\text{M}$). Open column = CGRP alone; solid column = CGRP + L-NOARG ($100 \mu\text{M}$); narrow hatched column = CGRP + L-NMMA ($10 \mu\text{M}$); wide hatched column = CGRP + L-NMMA ($100 \mu\text{M}$). Columns represent the means of 4–6 separate experiments with vertical bars indicating s.e.mean.

Figure 4 Concentration-response curves showing vasodilator responses (maximum height of response) of rat skin microvasculature to calcitonin gene-related peptide (circles) and acetylcholine (triangles) in the absence (closed symbols) and presence (open symbols) of N^G -nitro-L-arginine. Each symbol represents the mean of 5 separate experiments. *Denotes a significant difference from control.

Figure 5 Representative trace showing the effect of N^G -nitro-L-arginine (L-NOARG) or N^G -monomethyl-L-arginine (L-NMMA) (both at $100 \mu\text{M}$) on the vasodilator response to A23187 ($100 \mu\text{M}$ perfused for 10 min). Unbroken line = A23187 alone; dashed line = A23187 + L-NMMA; dashed-dotted line = A23187 + L-NOARG. L-NOARG or L-NMMA was introduced 14 min before and during perfusion with A23187. Point of addition of A23187 indicated by arrow.

Figure 6 Effect of N^G-nitro-L-arginine (L-NOARG) or N^G-monomethyl-L-arginine (L-NMMA) (both at 100 μ M) on the dilatation response of the rat skin microvasculature to A23187 (100 μ M). Open column = A23187 alone; solid column = A23187 + L-NOARG; hatched column = A23187 + L-NMMA. Columns represent the means of 5–6 separate experiments with vertical bars indicating s.e.mean. *Indicates significant difference from control.

Figure 7 Effect of capsaicin pretreatment and N^G-nitro-L-arginine (L-NOARG, 100 μ M) on the dilatation response of rat skin microvasculature to acetylcholine (ACh; 100 μ M). Open columns = ACh alone; solid columns = ACh + L-NOARG. Columns represent the means of 6–7 separate experiments with vertical bars indicating s.e.mean. *Indicates significant difference between responses obtained to ACh in capsaicin-treated rats and control rats. Control group: ^xindicates significant difference from control. Capsaicin-treated group: [†]indicates significant difference from capsaicin-control.

Figure 8 Effect of capsaicin pretreatment and N^G-nitro-L-arginine (L-NOARG, 100 μ M) on the dilatation response to calcitonin gene-related peptide (CGRP; 1 μ M). Open columns = CGRP alone; solid columns = CGRP + L-NOARG. Columns represent the means of 5–7 separate experiments with vertical bars indicating s.e.mean.

Effect of L-NOARG on vasodilator responses to ACh and CGRP in capsaicin pretreated rats

L-NOARG (100 μ M) significantly reduced the vasodilator response to ACh in capsaicin pretreated rats (Figure 7). This was primarily a reflection of a reduction in the time-dependent maintenance of the response since the maximum height of the response was not significantly affected.

L-NOARG had no significant effect on the vasodilator response to CGRP in capsaicin pretreated animals (Figure 8).

Discussion

In this study we have shown that in blister bases raised in rat skin, part of the vasodilator action of ACh is mediated via the generation of NO. A further component of the vasodilator response to ACh results following excitation of capsaicin-sensitive C-fibres which release a transmitter(s) that acts largely through NO-independent mechanisms. The vasodilator action of CGRP proceeds independently of both NO and primary sensory afferents.

In man, iontophoresed ACh can stimulate peripheral nociceptive C-fibres to produce flare (neurogenic vasodilatation) which is abolished by anaesthetic and is absent in denervated skin (Westerman *et al.*, 1987; Parkhouse & LeQuesne, 1988). A direct action of ACh on non-myelinated C-fibres has also been demonstrated in the saphenous nerve (Douglas & Ritchie, 1960) and cutaneous afferent nerves (Fjallbrandt & Iggo, 1961) of the cat. In the rabbit, nociceptive effects of acetylcholine have been shown to be mediated through nicotinic receptors present on perivascular sensory nerve endings (Juan, 1982). In the present study, capsaicin pretreatment was used to destroy selectively sensory nerves (Jancso *et al.*, 1977) and this significantly reduced ACh-induced vasodilator responses of the skin microvasculature. To our knowledge this is the first demonstration that ACh has an excitatory action on peripheral nociceptive C-fibres in the rat. Non-specific effects of capsaicin treatment were not likely to contribute since vasodilator responses to CGRP were unaffected.

Neurogenic inflammation may involve cholinergic nerves since atropine reduces axon reflex vasodilatation and plasma extravasation in rat skin (Couture *et al.*, 1985; Low & Westerman, 1989) and these nerves are likely to be of sympathetic postganglionic origin (Lembeck & Holzer, 1979; Couture *et al.*, 1985). In a previous study (Ralevic *et al.*, 1991) we suggested that NO may be involved in microvascular inflammatory responses, since L-NOARG attenuated substance P-induced vasodilatation and plasma extravasation. The present study provides further evidence to support this since L-NOARG and L-NMMA attenuated the vasodilator response to ACh. That the action of these inhibitors was specific was confirmed by the fact that the inactive stereoisomer, D-NMMA, was ineffective as an inhibitor of ACh-induced vasodilatation. Furthermore, neither L-NOARG nor L-NMMA inhibited the vasodilator response to CGRP, which is consistent with previous findings that CGRP generally does not activate the endothelium. Endothelium-dependent relaxations to CGRP have been demonstrated in some human vessels (Thom *et al.*, 1987) and in the rat aorta (Brain *et al.*, 1985; Grace *et al.*, 1987; Gray & Marshall, 1990).

The major source of NO in our system is probably the microvascular endothelium. However, it is also possible that NO may derive partly from mast cells (Salvemini *et al.*, 1990) since both A23187 and ACh have been shown to act on isolated mast cells, causing the release of histamine which is known to have potent effects on vascular permeability (Blandina *et al.*, 1980; Khalil & Helme, 1989). If an ACh- or A23187-induced release of NO from mast cells does occur in our system, this is likely to occur at concentrations of these agents below those required to induce the release of hista-

mine, since neither ACh nor A23187 elicited plasma extravasation (unpublished observations).

A comparison of concentration-response curves to CGRP and ACh showed the rat skin microvasculature to be approximately 100 fold more sensitive to CGRP than to ACh. While our results demonstrate an action of ACh on sensory nerves the largest proportion of its effects are mediated postjunctionally. Smooth muscle cells acting as a barrier to diffusion of ACh to receptors on endothelial cells and rapid hydrolysis of ACh could account for much of its lower potency compared with CGRP which, in our preparation, acts directly on receptors on the smooth muscle. In this respect it has been shown that in large blood vessels ACh is approximately 100 fold less sensitive when applied abluminally (Angus *et al.*, 1983). The implication of this is that sensory nerves releasing CGRP (together with substance P and other tachykinins) are comparatively more important than cholinergic fibres in neurogenic inflammation. The latter may have a role when relatively high local concentrations of ACh are achieved, possibly in severe cases of neurogenic trauma.

The most marked inhibitory effect of L-NOARG and L-NMMA on the vasodilator response to ACh was an attenuation of the time-dependent maintenance of the response. Similar effects have been observed in rats and guinea-pigs *in vivo* where L-NMMA (Aisaka *et al.*, 1989) and NOLA (synonymous with L-NOARG) (Zambetis *et al.*, 1991) attenuated the duration but not the amplitude of the hypotensive response to ACh. These workers suggested that the immediate vasodilatation of resistance vessels to ACh occurs by a mechanism which does not involve *de novo* synthesis of endothelially-derived NO, while the succeeding longer-lasting vasodilatation does depend on NO biosynthesis and the availability of L-arginine. Substantial components of the vasodilator response to ACh have also been shown to be resistant to inhibition by L-NMMA and L-NAME (the methyl ester of L-NOARG) in rabbit muscle microvasculature *in vivo* (Persson *et al.*, 1991), and human subcutaneous resistance arteries (Woolfson & Poston, 1990), while the vasodilator response to ACh was unaffected by L-NAME in conscious rats (Gardiner *et al.*, 1990). It is possible that hyperpolarization might be involved in the vasodilator action of ACh (Feletou & Vanhoutte, 1988; Chen & Suzuki, 1990) although it is not likely to be via the ATP-sensitive K⁺ channel since dilatation to ACh in the rat *in vivo* was not attenuated by glibenclamide (Zambetis *et al.*, 1991). A direct action on the vascular smooth muscle as has been observed in some vessels (Brayden & Bevan, 1985) also cannot be

excluded. At the highest concentration of ACh used (1 mM) the attenuation of the maximum height of the vasodilatation to ACh by L-NOARG indicates a significant contribution of NO to this part of the response. If multiple mechanisms are involved in the vasodilator response to ACh it is likely that their relative contributions vary with the concentration of agonist.

In capsaicin-treated rats, L-NOARG virtually abolished ACh-induced vasodilatation in contrast to control animals where the L-NOARG-resistant component constituted about 40% of the total ACh response. Since a substantial L-NOARG-resistant component was not observed after destruction of sensory nerves it is likely that the NO-independent component of the vasodilator response to ACh is largely due to the action of a mediator released from capsaicin-sensitive C-fibres. This mediator would not be expected to affect vascular permeability, since ACh did not elicit plasma extravasation. Further studies are warranted to identify the mediator released by ACh from sensory nerves which appears to act independently of NO. One of many possible candidates is CGRP which is localized in and released from sensory nerves (Gulbenkian *et al.*, 1986; Manzini *et al.*, 1991) and our experiments using L-NOARG confirmed the endothelium-independent nature of CGRP-induced vasodilatation in rat skin microvasculature.

In conclusion, we have shown that in rat skin microvasculature, the vasodilator response to ACh is comprised of two distinct components; one part of the response is mediated through NO probably arising following direct stimulation of the microvascular endothelium, and the other part of the response is mediated through an excitatory action on peripheral nociceptive C-fibres with release of a sensory transmitter which causes vasodilatation independently of NO. The vasodilator response to CGRP is independent of NO and sensory nerves. It has previously been shown that plasma protein extravasation induced by substance P in rat skin is reduced by atropine suggesting cholinergic modulation of this response (Couture & Kerouac, 1987). These data, together with the present results, suggests that reciprocal interactions between cholinergic and sensory nerve fibres may contribute to the rapid spread of the inflammatory response.

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