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Mediation by prostaglandins of the nitric oxide-induced neurogenic vasodilatation in rat skin

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1 Intraplantar administration of the nitric oxide (NO) donor, sodium nitroprusside (SNP), induces hyperaemia in the rat paw skin, which is in part due to release of calcitonin gene-related peptide (CGRP) from afferent nerve fibres. The present study examined whether prostaglandins or other inflammatory mediators participate in the neurogenic vasodilatation caused by SNP. Blood flow in the plantar hindpaw skin of urethane-anaesthetized rats was measured by laser Doppler flowmetry.

2 The hyperaemic responses to intraplantar administration of the NO donors SNP (150 pmol) and 3-morpholino-sydnonimine (SIN-1, 15 nmol) were attenuated by 45% and 61%, respectively, after injection of the CGRP antagonist, $CGRP_{8-37}$ (50 nmol kg^{-1}, i.v.) which did not significantly change baseline blood flow.

3 The NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 15 mg kg^{-1}, i.v.), the bradykinin antagonist Hoe-140 (100 nmol kg^{-1}, i.v.) and the histamine antagonists, pyrilamine (2 mg kg^{-1}, i.v.) plus cimetidine (10 mg kg^{-1}, i.p.) were without effect on baseline blood flow and the vasodilatation caused by SNP.

4 The cyclo-oxygenase inhibitors, indomethacin (10 mg kg^{-1}, i.p.) and flurbiprofen (5 mg kg^{-1}, i.p.) depressed the SNP-induced hyperaemia by 65% and 42%, respectively, without altering baseline blood flow. The ability of $CGRP_{8-37}$ to inhibit the vasodilator response to SNP was lost in indomethacin-treated rats.

5 Intraplantar administration of prostaglandin E_2 (PGE_2, 15 pmol) evoked cutaneous vasodilatation which was attenuated by 66% after administration of $CGRP_{8-37}$ but remained unaltered by indomethacin or L-NAME.

6 These data indicate that the neurogenic hyperaemia which in rat skin is induced by intraplantar administration of NO donors involves the formation of prostaglandins which in turn cause release of the vasodilator peptide, CGRP, from perivascular afferent nerve fibres.

Keywords: Nitric oxide; N^G-nitro-L-arginine methyl ester (L-NAME); sodium nitroprusside; 3-morpholine-sydnonimine (SIN-1); prostaglandin E_2; indomethacin; calcitonin gene-related peptide (CGRP); afferent nerve fibres; skin blood flow; neurogenic vasodilatation

Introduction

Chemical stimulation of afferent nerve fibres in the skin causes an inflammatory reaction that begins with a marked rise in cutaneous blood flow. The major mediator of this neurogenic vasodilatation is thought to be calcitonin gene-related peptide (CGRP) which is released from afferent nerve fibres and which is a very potent vasodilator substance (Delay-Goyet *et al.*, 1992; Escott & Brain, 1993; Hughes & Brain, 1994). It has recently been found that another vasodilator mediator, nitric oxide (NO), does not play a vasorelaxant messenger role in neurogenic vasodilatation but can contribute to the excitation of, or peptide release from, afferent nerve fibres (Holzer & Jocič, 1994; Hughes & Brain, 1994; Kajekar *et al.*, 1995). This concept has been substantiated by the obesrvation that the cutaneous hyperaemia caused by the NO donor, sodium nitroprusside (SNP), is in part inhibited by the specific CGRP antagonist $CGRP_{8-37}$ and by capsaicin-induced defunctionalization of afferent nerve fibres (Holzer & Jocič, 1994). This and the finding that $CGRP_{8-37}$ is no longer able to inhibit the vasodilator effect of SNP in capsaicin-pretreated rats (Holzer & Jocič, 1994) indicate that SNP can dilate blood vessels in rat skin by releasing CGRP from afferent nerve fibres, a conclusion that also holds true for the SNP-evoked dilatation of cerebral arterioles in the cat (Wei *et al.*, 1992).

The conjecture that NO can stimulate the release of vasodilator peptides from afferent nerve fibres raises the question as to the mechanism by which NO excites afferent nerves. Important in this context are the findings that NO is able to stimulate the formation of prostaglandins in the rat hindpaw (Sautebin *et al.*, 1995) and to enhance the plasma concentration of 6-keto $PGF_{1\alpha}$ (Salvemini *et al.*, 1995) and that prostaglandins can facilitate the release of CGRP and other peptides from afferent neurones (Geppetti *et al.*, 1991; Andreeva & Rang, 1993; Hingtgen & Vasko, 1994; Hua *et al.*, 1994; Vasko *et al.*, 1994). A more direct line of evidence for an interaction of NO and prostaglandins in the activation of afferent vasodilator nerves comes from studies of the effect of interleukin-1β on the neurogenic vasodilatation evoked by capsaicin in rat skin (Herbert & Holzer, 1994a,b). The ability of this cytokine to augment the capsaicin-induced hyperaemia by a prejunctional site of action is prevented not only by blockade of NO synthesis with N^G-nitro-L-arginine methyl ester (Herbert & Holzer, 1994b) but also by the cyclo-oxygenase inhibitor, indomethacin (Herbert & Holzer, 1994a).

With this background information it was the aim of the present study to confirm that SNP and a chemically different NO donor are able to evoke neurogenic CGRP-mediated vasodilatation in rat skin, to examine whether prostaglandins participate in this action of SNP and whether prostanglandins themselves can induce a CGRP-mediated hyperaemia, and thereby to elucidate the sequential or parallel mechanism by which NO and prostaglandins interact in the excitation of afferent vasodilator nerves. In addition we investigated whether other intermediate messengers such as bradykinin or histamine are involved in the neurogenic vasodilator response to SNP.

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Methods

Experimental procedures

The experiments of this study were approved by an animal experiment committee at the Austrian Ministry of Science and Research. Female Sprague-Dawley rats (Versuchstierzuchtanstalt Himberg, Austria) weighing 180–220 g were anaesthetized with urethane, 1.5 g kg^{-1}, s.c., placed on a heated table to maintain their rectal temperature at 37–38°C, and fitted with a tracheal cannula. A catheter in the left jugular vein was used for the continuous infusion of saline (1.5 ml h^{-1}), to avoid dehydration, and for the i.v. administration of drugs which were given in volumes of 1 ml kg^{-1}. Mean arterial blood pressure (MAP) was recorded from a catheter inserted in the right carotid artery and connected to a pressure transducer.

For intraplantar administration of drugs a catheter fitted with an injection needle (outside diameter 0.3 mm) was inserted from the distal aspect of the hindpaw into the middle of the plantar subcutaneous space. Substances were administered by infusion of a volume of 15 μl over a period of 1 min with an infusion pump. Thus it was possible to record blood flow continuously, without injection artifacts, during and after the intraplantar administration of drugs.

Cutaneous blood flow was recorded with a laser Doppler flowmeter (model MBF3D, Moor Instruments, Devon, U.K.). To this end, a stainless steel laser optic probe (model P1, Moor Instruments) was taped perpendicularly on the centre of the plantar side of the hindpaw in close proximity to the tip of the infusion needle. After completion of the experimental preparations an equilibration period of 20 min was allowed before the experiments were begun. Blood flow was monitored as flux (no dimension), the time constant being 0.5 s, and changes in blood flow were expressed as percentage changes in flux (Escott & Brain, 1993; Holzer & Jocič, 1994) relative to the average values recorded during the 2 min-period immediately before the changes were induced.

Experimental protocols

Up to three successive applications of SNP or 3-morpholino-sydnonimine (SIN-1) were made in each hindpaw, intervals of at least 20 min being allowed between the intraplantar infusions of the NO donors. The blood flow response to the first administration of SNP or SIN-1, which was sometimes erratic (Holzer & Jocič, 1994), was not evaluated and the response to the second application of the NO donors was taken as the reference response.

When the effects of $CGRP_{8-37}$, L-NAME, methylene blue, Hoe-140 or pyrilamine plus cimetidine were examined, the study design was to compare the vasodilator response to SNP or SIN-1 recorded after the respective treatments with the reference response recorded beforehand. The NO donors, SNP or SIN-1, were first administered twice into the right hindpaw, after which the respective vehicle was injected systemically and a third application of the NO donor was made. Then SNP or SIN-1 was administered twice into the left hindpaw, after which $CGRP_{8-37}$, L-NAME, methylene blue, Hoe-140 or pyrilamine plus cimetidine was given systemically before a third blood flow response to SNP or SIN-1 was recorded. The precise intervals between the injection of the drugs or their vehicle and the administration of the NO donors are specified in the Results section.

In the experiments designed to test the effect of flurbiprofen and indomethacin on the vasodilator reaction to SNP, the study design was to compare the SNP-induced reference response determined in vehicle-treated rats with that seen in the drug-treated animals. Forty min after the i.p. injection of flurbiprofen or its vehicle, the first application of SNP into the left hindpaw was made, which 20 min later was followed by a second application of SNP to record the reference blood flow response. The experiments involving indomethacin were carried out with a similar protocol. In addition, after recording the reference blood flow response to SNP, the NO donor was administered a third time into the left hindpaw, $CGRP_{8-37}$ being injected 2 min before the third application of SNP.

PGE_2 was administered only once to each hindpaw because of the slow reversibility of its vasodilator action. In a first study, L-NAME or indomethacin was administered systemically 15 or 60 min, respectively, before the intraplantar infusion of PGE_2 into the left hindpaw. The systemic administration of the respective vehicle solutions followed the same protocol. In a second study, PGE_2 was first administered into the right hindpaw after vehicle (saline) had been injected i.v. 2 min beforehand. Thirty minutes later PGE_2 was infused into the left hindpaw 2 min after $CGRP_{8-37}$ or its vehicle had been given i.v.

Statistics

All data are expressed as means ± s.e.mean. Statistical evaluation of the results was performed with the Mann-Whitney U tests, when two independent groups of data were compared with each other, or with the Kruskal-Wallis H test followed by the Mann-Whitney U test, when more than two independent groups of data were compared with each other. The Wilcoxon test for pair differences was used when data obtained before and after drug treatment were compared with each other. Probability values $P<0.05$ were regarded as significant.

Substances and solutions

The saline solution used was 0.9% NaCl (w.t./w.t.). Saline was used to dissolve human α-calcitonin gene-related peptide-(8-37) ($CGRP_{8-37}$; 0.5 mM; Bachem, Bubendorf, Switzerland), Hoe-140 (icatibant; 1 mM; Hoechst, Frankfurt am Main, Germany), methylene blue (100 mM; Serva, Heidelberg, Germany), N^G-nitro-L-arginine methyl ester (L-NAME; 15 mg ml^{-1}; Bachem, Bubendorf, Switzerland), pyrilamine maleate (2 mg base ml^{-1}; Sigma, Vienna, Austria), and sodium nitroprusside (SNP; 1 mM Merck, Darmstadt, Germany). Further dilutions of the stock solutions, if required, were also made with saline.

Cimetidine (Smith Kline & French, Welwyn Garden City, Herts, U.K.) was dissolved in 0.1 M HCl followed by neutralization with NaOH, the final solution consisting of 10 mg ml^{-1} cimetidine, 35 mM HCl, 35 mM NaOH and 45 mM phosphate buffer pH 7.4. This solution was administered i.p. at a volume of 1 ml kg^{-1}. Racemic flurbiprogen (Sigma, Vienna, Austria) was dissolved in 0.03 M phosphate buffer pH 7.4 at a concentration of 2.5 mg ml^{-1}, this solution being injected i.p. at a volume of 2 ml kg^{-1}. Indomethacin (Sigma, Vienna, Austria) was dissolved in 2% (w.t./w.t.) Na_2CO_3 at a concentration of 30 mg ml^{-1} and further diluted with saline to give a 10 mg ml^{-1} solution which was injected i.p. at a volume of 1 ml kg^{-1}. 3-Morpholino-sydnonimine hydrochloride (SIN-1; 1 mM; Cassella, Frankfurt am Main, Germany) was dissolved in water at a concentration of 10 mM and diluted with saline to give a 1 mM infusion solution. Prostaglandin E_2 (PGE_2; Sigma, Vienna, Austria) was dissolved in absolute ethanol at a concentration of 1 mM and diluted 1:1,000 in one step with saline to obtain a 1 μM infusion solution. Urethane (Fluka, Buchs, Switzerland) was dissolved in water at a concentration of 25% (w.t./w.t.) and injected s.c. at a volume of 6 ml kg^{-1}.

Results

Effect of $CGRP_{8-37}$ on the cutaneous hyperaemia induced by SNP and SIN-1

The doses of sodium nitroprusside (SNP) and 3-morpholino-sydnonimine (SIN-1) to be used were determined in pilot studies in which the intraplantar doses of 15 pmol SNP and 1.5 nmol SIN-1 were found to be threshold in increasing cu-

taneous blood flow. Basal blood flow in these pilot experiments was 49 ± 2.4 (flux without dimension, $n=6$), 15 pmol SNP enhancing cutaneous blood flow by 15 ± 4.9% ($n=3$) and 1.5 nmol SIN-1 increasing cutaneous blood flow by 19 ± 7.6% ($n=3$). Tenfold higher doses of each NO donor were administered in all subsequent experiments. Figure 1 shows that 150 pmol SNP (15 μl of a 10 μM solution) caused a considerably higher rise in cutaneous blood flow than 15 nmol SIN-1 (15 μl of a 1 mM solution). As reported previously (Holzer & Jocič, 1994), the hyperaemic response to SNP was transient and did not last for more than 10 min, a time course that was also true for the vasodilatation caused by SIN-1. Intraplantar administration of 15 μl saline (the vehicle for the NO donors) failed to significantly enhance blood flow (+12 ± 6.0%, $n=9$, $P>0.05$; Holzer & Jocič, 1994), and saline ($n=9$) SNP ($n=6$) or SIN-1 ($n=6$) had no appreciable effect on mean arterial blood pressure (MAP, 89 ± 2.6 mmHg before saline, 91 ± 2.0 mmHg after saline, 96 ± 5.7 mmHg before SNP, 95 ± 5.5 mmHg after SNP, 85 ± 3.2 mmHg before SIN-1, 87 ± 1.3 mmHg after SIN-1).

The CGRP antagonist $CGRP_{8-37}$ (50 nmol kg^{-1}; Delay-Goyet *et al.*, 1992; Holzer & Jocič, 1994) or its vehicle (saline, 1 ml kg^{-1}) was injected i.v. 2 min before the third intraplantar application of SNP or SIN-1. Both $CGRP_{8-37}$ ($n=12$) and it vehicle ($n=12$) failed to change significantly cutaneous blood flow (52 ± 8.0 before $CGRP_{8-37}$, 58 ± 8.2 after $CGRP_{8-37}$, 44 ± 4.9 before vehicle, 46 ± 4.9 after vehicle) and MAP (93 ± 3.5 mmHg before $CGRP_{8-37}$, 94 ± 3.4 mmHg after $CGRP_{8-37}$, 92 ± 4.1 mmHg before vehicle, 93 ± 4.0 mmHg after vehicle). The SNP-induced hyperaemia was attenuated by 45% after administration of $CGRP_{8-37}$ but was left unaltered by the vehicle (Figure 1). The ability of intraplantar SIN-1 to augment cutaneous blood flow was attenuated by 61% in rats that had received $CGRP_{8-37}$ (Figure 1).

Effect of L-NAME and methylene blue on the cutaneous hyperaemia induced by SNP

The inhibitor of NO synthase, N^G-nitro-L-arginine methyl ester (L-NAME, 15 mg kg^{-1}; Holzer & Jocič, 1994), or its vehicle (saline, 1 ml kg^{-1}) was injected i.v. 15 min before the third intraplantar application of SNP (150 pmol). Unlike the vehicle, L-NAME caused a prompt and sustained increase in MAP (86 ± 2.4 mmHg before vehicle, 88 ± 3.0 mmHg after vehicle, 87 ± 3.0 mmHg before L-NAME, 136 ± 4.8 mmHg after L-NAME, $P<0.01$, $n=6$ for all groups), while both the vehicle and L-NAME failed to alter significantly cutaneous blood flow (51 ± 7.6 before vehicle, 60 ± 10.4 after vehicle, 59 ± 4.8 before L-NAME, 58 ± 7.2 after L-NAME, $n=6$ for all groups) or the SNP-evoked hyperaemia (Figure 2).

The inhibitor of soluble guanylate cyclase, methylene blue (3 μmol in 30 μl; Duarte *et al.*, 1990), was administered by intraplantar infusion 40 min before the third intraplantar application of SNP (150 pmol). Methylene blue had no effect on MAP (91 ± 3.1 mmHg before methylene blue, 89 ± 2.9 mmHg after methylene blue) but immediately caused a huge rise in cutaneous blood flow to 419 ± 73% of control ($n=6$). Forty min post-methylene blue, blood flow had come down to 141 ± 13% of control ($n=6$), and at this point in time the hyperaemia caused by SNP was depressed by 44% (Figure 2).

Effect of bradykinin and histamine receptor antagonists on the cutaneous hyperaemia induced by SNP

The bradykinin B_2 receptor antagonist, Hoe-140 (100 nmol kg^{-1}; Lembeck *et al.*, 1991) or its vehicle (saline, 1 ml kg^{-1}) was injected i.v. 15 min before the third intraplantar application of SNP (150 pmol). Neither the vehicle nor Hoe-140 caused any significant change in MAP (91 ± 4.1 mmHg before vehicle, 90 ± 3.3 mmHg after vehicle, 99 ± 4.4 mmHg before Hoe-140, 100 ± 3.9 mmHg after Hoe-140, $n=5$ for all groups) and cutaneous blood flow (63 ± 20.4 before vehicle, 58 ± 18.0 after vehicle, 52 ± 14.2 before Hoe-140, 57 ± 14.6 after Hoe-140, $n=5$ for all groups). The SNP-evoked hyperaemia did not differ between rats treated with the vehicle (+127 ± 24.7%, $n=5$) and those treated with Hoe-140 (+140 ± 34.7%, $n=5$).

The histamine H_1 receptor antagonist, pyrilamine (2 mg kg^{-1}, i.v.; Hahn, 1978) plus the histamine H_2 receptor antagonist, cimetidine (10 mg kg^{-1}, i.p.; Brimblecombe *et al.*, 1975; Couture & Cuello, 1984) or their respective vehicle solutions were administered 15 min before the third intraplantar application of SNP. Both the vehicle and pyrilamine plus cimetidine failed to alter MAP significantly (88 ± 5.2 mmHg before vehicle, 89 ± 4.2 mmHg after vehicle, 92 ± 4.4 mmHg before pyrilamine plus cimetidine, 93 ± 4.1 mmHg after pyrilamine plus cimetidine, $n=5$ for all groups) or cutaneous blood flow (28 ± 6.0 before vehicle, 31 ± 6.0 after vehicle, 36 ± 6.7 before pyrilamine plus cimetidine, 53 ± 14.0 after pyrilamine plus cimetidine, $n=5$ for all groups). The ability of SNP to cause cutaneous vasodilatation was not different in rats treated with the vehicle (+178 ± 45.2%, $n=5$) and those treated with pyrilamine plus cimetidine (+166 ± 41.6%, $n=5$).

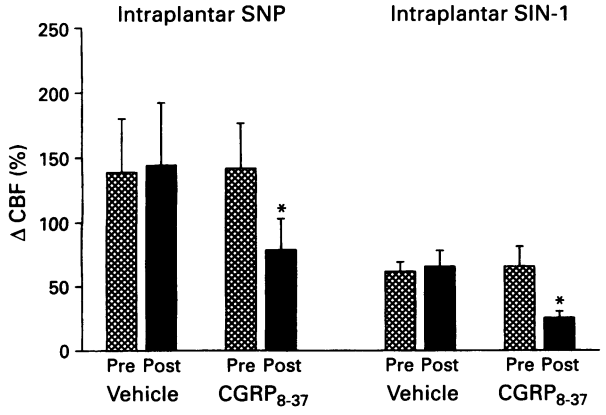

Figure 1 Increment of cutaneous blood flow (Δ CBF) evoked by intraplantar administration of sodium nitroprusside (SNP, 150 pmol) or 3-morpholino-sydnonimine (SIN-1, 15 nmol) in rats treated with i.v. vehicle (1 mg kg^{-1} saline) or i.v. $CGRP_{8-37}$ (50 nmol kg^{-1}). The responses 'pre'-treatment were evoked 20 min before, the responses 'post'-treatment 2 min after the injection of $CGRP_{8-37}$ or its vehicle. The values shown are means with s.e.mean; $n=6$. $^*P<0.05$ versus respective 'pre'-treatment value (Wilcoxon test for pair differences).

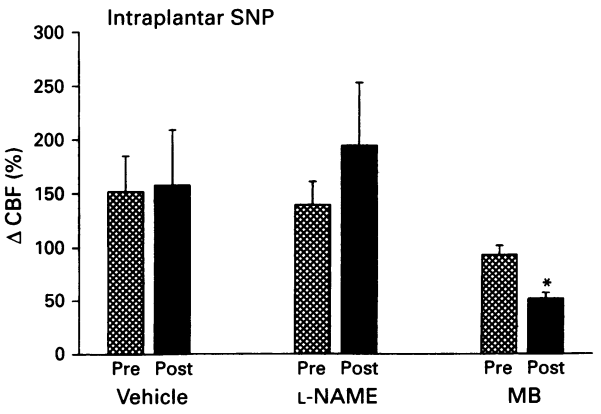

Figure 2 Increment of cutaneous blood flow (Δ CBF) evoked by intraplantar administration of sodium nitroprusside (SNP, 150 pmol) in rats treated with i.v. vehicle, i.v. N^G-nitro-L-arginine methyl ester (L-NAME, 15 mg kg^{-1}) or intraplantar methylene blue (MB, 3 μmol). The responses to SNP 'pre'-treatment were evoked 10 min before, the responses 'post'-treatment 15 min (vehicle, L-NAME) or 40 min (MB) after the treatments. The values shown are means with s.e.mean; $n=6$. $^*P<0.05$ versus respective 'pre'-treatment value (Wilcoxon test for pair differences).

Effect of flurbiprofen and indomethacin on the cutaneous hyperaemia induced by SNP

The cyclo-oxygenase inhibitors flurbiprofen (5 mg kg^{-1}; Peskar *et al.*, 1991) and indomethacin (10 mg kg kg^{-1}; Couture & Cuello, 1984; Dembinska-Kiec *et al.*, 1991) or their respective vehicle solutions were injected i.p., 60 min before the second intraplantar applications of SNP (150 pmol). Both flurbiprofen and indomethacin failed to alter cutaneous blood flow significantly (59 ± 7.6, $n=8$, in flurbiprofen-treated rats, 40 ± 9.0, $n=8$, in rats treated with the vehicle for flurbiprofen; 70 ± 8.5; $n=7$, in indomethacin-treated rats, 53 ± 7.6, $n=6$, in rats treated with the vehicle for indomethacin) or MAP (89 ± 2.4 mmHg, $n=8$, in flurbiprofen-treated rats, 91 ± 3.2 mmHg, $n=8$ in rats treated with the vehicle for flurbiprofen, 85 ± 4.0 mmHg, $n=7$, in indomethacin-treated rats, 87 ± 7.4 mmHg, $n=6$, in rats treated with the vehicle for indomethacin). As can be seen from Figure 3, the SNP-evoked rise in cutaneous blood flow in flurbiprofen-treated rats was attenuated to 58% of that seen in vehicle-treated rats, and the SNP-induced hyperaemia in indomethacin-treated rats was reduced to 35% of that measured in vehicle-treated rats. Further experiments showed that the ability of $CGRP_{8-37}$ to depress the SNP-induced rise in cutaneous blood flow was lost in indomethacin-treated rats. While in vehicle-treated rats $CGRP_{8-37}$ (50 nmol kg^{-1} injected i.v. 2 min before the third intraplantar application of SNP) inhibited the vasodilator response to SNP by 44%, the CGRP antagonist was no longer able to reduce the SNP-evoked hyperaemia in indomethacin-treated animals (Figure 3).

Effect of prostaglandin E_2 on cutaneous blood flow

The dose of prostaglandin E_2 (PGE_2) used was determined in pilot experiments in which the intraplantar administration of 1.5 pmol PGE_2 was found to be threshold in increasing cutaneous blood flow ($+23 \pm 9.6\%$, $n=3$). All subsequent tests were carried out with a tenfold higher dose of PGE_2 (15 pmol) which caused a marked and sustained increase in cutaneous blood flow (Figure 4), an effect that often lasted for more than 45 min. A comparison of the MAP values measured before and after administration of PGE_2 in the 4 vehicle groups illustrated in Figure 4 (87 ± 2.3 mmHg before PGE_2, 88 ± 2.4 mmHg after PGE_2, $n=24$) revealed that MAP was not changed by the prostaglandin. The ability of PGE_2 to increase cutaneous blood flow did not differ between rats that had been treated with L-NAME (15 mg kg^{-1} injected i.v. 15 min before the intraplantar application of PGE_2), indomethacin (10 mg kg^{-1} injected i.p. 60 min before administration of PGE_2) or their respective vehicle solutions (Figure 4). A further group of experiments demonstrated that $CGRP_{8-37}$ (50 nmol kg^{-1} injected i.v. 2 min before application of PGE_2) was able to inhibit the vasodilator response to PGE_2 by 66%, whereas the vehicle (1 ml kg^{-1} saline) did not alter the PGE_2-evoked hyperaemia significantly (Figure 4).

Discussion

The present findings confirm the notion (Holzer & Jocič 1994) that the cutaneous hyperaemia caused by the intraplantar application of the NO donor SNP involves CGRP, because it is significantly reduced by the specific CGRP antagonist $CGRP_{8-37}$. Together with the observation that the $CGRP_{8-37}$-sensitive component of the vasodilator response to SNP is abolished by capsaicin-induced defunctionalization of afferent, neurones (Holzer & Jocič, 1994), this finding provides strong evidence that part of the SNP-evoked rise in cutaneous blood flow is due to release of CGRP from afferent nerves fibres. A similar conclusion holds true for the ability of SNP to dilate cerebral arterioles in the cat (Wei *et al.*, 1992). The proposed role of CGRP in the vasodilator response to SNP is in keeping with the concept that neurogenic vasodilatation in rat and rabbit skin is mostly, if not exclusively, mediated by afferent nerve-derived CGRP (Delay-Goyet *et al.*, 1992; Escott & Brain, 1993; Hughes & Brain, 1994).

The major aim of the present study was to elucidate the mechanisms by which SNP stimulates afferent nerve fibres to release vasodilator quantities of CGRP in rat skin, and the first objective in this respect was to examine whether NO liberated from SNP or another property of the molecule was responsible for the neurogenic hyperaemia caused by this NO donor. The

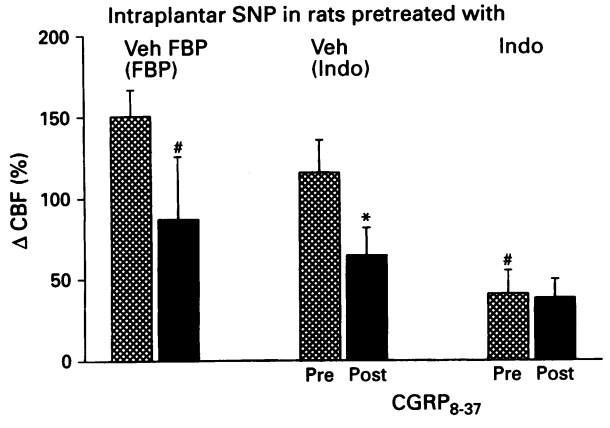

Figure 3 Increment of cutaneous blood flow (Δ CBF) evoked by intraplantar administration of sodium nitroprusside (SNP, 150 pmol) in rats treated with i.p. vehicle for flurbiprofen [Veh (FBP), $n=8$], i.p. flurbiprofen (FBP, 5 mg kg^{-1}, $n=8$) i.p. vehicle for indomethacin [Veh (Indo), $n=6$] or i.p. indomethacin (Indo, 10 mg kg^{-1}, $n=7$) 60 min beforehand. In the rats injected with indomethacin or its vehicle the responses 'pre'-treatment were evoked 20 min before, and the responses 'post'-treatment 2 min after, the i.v. injection of $CGRP_{8-37}$ (50 nmol kg^{-1}). The values shown are means with s.e.mean; n as indicated above. $^{*}P<0.05$ versus respective 'pre'-treatment value (Wilcoxon test for pair differences); $\#P<0.05$ versus respective value obtained in vehicle-treated rats (Mann-Whitney U test, partly in conjunction with the Kruskal-Wallis H test).

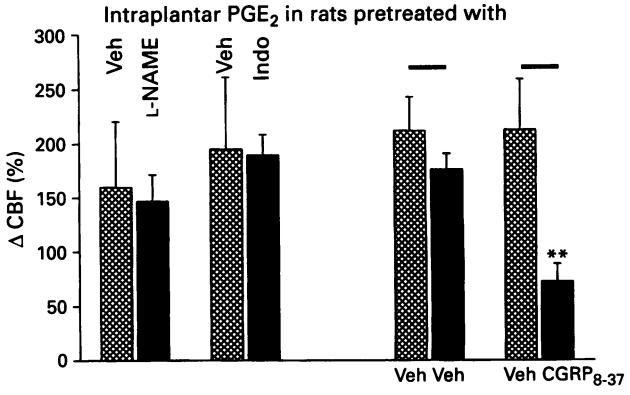

Figure 4 Increment of cutaneous blood flow (Δ CBF) evoked by intraplantar administration of prostaglandin E_2 (PGE_2, 15 pmol) in rats pretreated with i.v. N^G-nitro-L-arginine methyl ester (L-NAME, 15 mg kg^{-1}, $n=8$) or its vehicle (Veh, $n=8$) 15 min beforehand, in rats pretreated with i.p. indomethacin (Indo, 10 mg kg^{-1}, $n=5$) or its vehicle (Veh, $n=5$) 60 min beforehand, and in untreated (–) rats. In the untreated rats PGE_2 was first administered into the right hindpaw (cross-hatched column) 2 min after i.v. injection of vehicle (Veh) and then given into the left hindpaw (solid columns) 2 min after i.v. injection of vehicle (Veh, $n=6$) or $CGRP_{8-37}$ (50 nmol kg^{-1}, $n=5$). The values shown are means with s.e.mean; n as indicated above. $^{**}P<0.01$ versus respective vehicle value (Mann-Whitney U test).

observation that SIN-1, a NO donor which is chemically very different from SNP, was likewise able to increase blood flow in a $CGRP_{8-37}$-sensitive manner strongly suggests that NO is the active principle liberated from SNP and SIN-1, which in turn causes neurogenic vasodilatation in rat skin. Compared to SNP, SIN-1 appeared to be less potent and effective in raising cutaneous blood flow, which is in line with its relatively low relaxant activity in other vascular beds (Thelen *et al.*, 1992). Further, albeit inconclusive, evidence for NO being the active entity came from the ability of methylene blue to attenuate the vasodilator response to SNP. This inference takes account of the ability of methylene blue to inhibit the activation of soluble guanylate cyclase and the subsequent formation of guanosine 3':5'-cyclic monophosphate, a second messenger system that is responsible for many biological actions of NO (Ignarro & Kadowitz, 1985; Moncada *et al.*, 1991). However, since intraplantar application of methylene blue at the active dose of 3 μmol (Duarte *et al.*, 1990) caused a marked and long-lasting hyperaemia of unknown mechanism, caution should be exercised in the pharmacological assessment of the actions of this compound. In additional experiments it was ruled out that the vasodilator activity of SNP depended on endogenous NO formed via the L-arginine; NO synthase pathway (Moncada *et al.*, 1991), since the SNP-evoked hyperaemia remained unaltered by an effective dose of L-NAME (15 mg kg^{-1}; Holzer & Jocič, 1994).

Having established that NO liberated from SNP (10 μM) is responsible for the neurogenic vasodilatation caused by this compound, the question arose as to whether SNP-derived NO can directly stimulate afferent nerve fibres to release the vasodilator peptide CGRP or whether intermediary messengers are involved in this action. Peripherally injected or synthesized NO has been reported to cause pain (Holthusen & Arndt, 1994) and to contribute to nociception (Kawabata *et al.*, 1994), an activity that is consistent with the concept of neurogenic vasodilatation being mediated by nociceptive afferent nerve fibres (Holzer, 1992). Although SNP (100 μM) can facilitate the stimulus-evoked release fo CGRP from sensory neurones (Dymshitz & Vasko, 1994) it is unable to release, on its own, CGRP from afferent nerve fibres in the rat spinal cord (Garry *et al.*, 1994) or rat afferent neurones grown in culture (Dymshitz & Vasko, 1994), unless exorbitant concentrations of 10–100 mM SNP are used (Garry *et al.*, 1994). This failure of SNP (100 μM) to release CGRP may indicate that NO does not directly act on afferent neurones but requires intermediary messengers that are formed in sufficient quantities in the skin but not in the spinal cord or in cultures of afferent neurones.

Inflammatory mediators that are formed or liberated in the tissue in response to trauma or irritation include bradykinin, histamine and prostaglandins (Dray *et al.*, 1994). Bradykinin excites afferent neurones via constitutively expressed bradykinin B_2 receptors (Geppetti, 1993; Dray *et al.*, 1994) but since an effective dose of the specific bradykinin B_2 receptor antagonist, Hoe-140 (Hock *et al.*, 1991; Lembeck *et al.*, 1991; Wirth *et al.*, 1991) did not alter the SNP-evoked hyperaemia, an involvement of this kinin can be ruled out. Histamine is also unlikely to be involved as judged from the lack of effect of the histamine H_1 receptor antagonist, pyrilamine and the histamine H_2 receptor antagonist, cimetidine, which were administered in combination. Histamine is known to activate afferent neurones primarily via histamine H_1 receptors (Dray *et al.*, 1994; Ohkubo *et al.*, 1995) while histamine H_3 receptors inhibit peptide release from these neurones (Ohkubo *et al.*, 1995)

By contrast, the cyclo-oxygenase inhibitor, indomethacin, at a dose (10 mg kg^{-1}) known to cause a near-maximal blockade of prostaglandin biosynthesis in the rat gastric mucosa (Dembinska-Kiec *et al.*, 1991), depressed that SNP-induced hyperaemia to a significant extent. This result implies that cyclo-oxygenase products such as prostaglandins are intermediary messengers of the neurogenic vasodilator response to SNP-derived NO. In an attempt to confirm this inference with a cyclo-oxygenase inhibitor that is of a chemically different class from indomethacin (Flower & Vane, 1974) it was found that flurbiprofen was able to attenuate the SNP-evoked rise in blood flow. The dose of flurbiprofen (5 mg kg^{-1}) used here had previously been shown to cause a nearly maximal depression of prostanoid formation in various rat tissues (Peskar *et al.*, 1991). Although indomethacin inhibited the vasodilator response to SNP to a larger degree than flurbiprofen it seems unlikely that indomethacin did so by a non-specific cyclo-oxygenase-unrelated action because it did not change baseline blood flow and failed to alter the vasodilatation caused by PGE_2.

The hypothesis that prostaglandins are intermediate messengers of SNP-derived NO in causing neurogenic vasodilatation was substantiated by a number of additional data and considerations. Thus, the ability of $CGRP_{8-37}$ to reduce the SNP-induced hyperaemia was lost in indomethacin-treated rats, which indicates that cyclo-oxygenase products of arachidonic acid metabolism are essential for the ability of NO to release vasodilator quantities of CGRP from afferent nerve fibres. The existence of such a sequential mechanism was further corroborated by the observation that the cutaneous vasodilatation induced by PGE_2 depends to a considerable extent on CGRP but does not involve the formation of endogenous NO or prostaglandins, as shown by the effects of $CGRP_{8-37}$, L-NAME and indomethacin on the vasodilator response to PGE_2. These results are, on the one hand, in keeping with the ability of prostaglandins to sensitize and excite nociceptive afferent neurones (Dray *et al.*, 1994), to cause release of CGRP from afferent nerve fibres in the guinea-pig heart (Geppetti *et al.*, 1991) and rat trachea (Hua *et al.*, 1994) and to facilitate the stimulus-evoked release of CGRP from afferent nerve terminals in the spinal cord (Andreeva & Rang, 1993) or from afferent neurones grown in culture (Hingtgen & Vasko, 1994; Vasko *et al.*, 1994). On the other hand, the proposed role of prostaglandins in mediating the SNP-evoked hyperaemia is in line with the reported ability of NO to stimulate the formation of prostaglandins in the rat hindpaw (Sautebin *et al.*, 1995) and to elevate the plasma concentration of 6-keto $GF_{1\alpha}$ (Salvemini *et al.*, 1995).

In conclusion, the present study has shown that intraplantar application of SNP causes hyperaemia in the rat hindpaw skin, which in part is brought about by the sequence of NO-mediated activation of cyclo-oxygenase, prostaglandin-mediated excitation of afferent nerve fibres and release of the vasodilator peptide, CGRP. This sequential mechanism is also likely to account for the ability of interleukin-1β to augment neurogenic vasodilatation in the skin, an effect that is prevented both by L-NAME (Herbert & Holzer, 1994b) and indomethacin (Herbert & Holzer, 1994a). It thus seems conceivable that the NO:-prostaglandin:afferent nerve-derived CGRP pathway is of pathophysiological importance in inflammatory processes that are driven by excess NO production which, for instance, takes place after sunburn (Deliconstantinos *et al.*, 1995) or immune challenge (Moncada *et al.*, 1991).

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