

Mechanisms of the cutaneous vasodilator response to local external pressure application in rats: involvement of CGRP, neurokinins, prostaglandins and NO

¹Bérendère Fromy, ¹Sandra Merzeau, ¹Pierre Abraham & ^{*,1}Jean-Louis Saumet

¹Laboratoire de Physiologie, Faculté de Médecine d'Angers, F-49045 Angers, cedex France

1 Local pressure-induced vasodilation (PIV) is a neural vasodilator response to non-nociceptive externally applied pressure in the skin, previously described in humans. We first determined whether PIV exists in rats and depends on capsaicin-sensitive fibres as it does in humans. We then examined the mediators involved in the efferent pathway of PIV.

2 Cutaneous blood flow was measured by laser Doppler flowmetry during 11.1 Pa s⁻¹ increases in local applied pressure in anaesthetized rats. The involvement of capsaicin-sensitive fibres in PIV was tested in rats treated neonatally with capsaicin. To antagonize CGRP, neurokinin-1, -2, or -3 receptors, different groups of rats were treated with CGRP₈₋₃₇, SR140333, SR48968 or SR142801, respectively. Prostaglandins involvement was tested with indomethacin treatment. To inhibit nitric oxide synthase (NOS) activity or specific neuronal NOS, rats were treated with N^G-nitro-L-arginine or 7-nitroindazole, respectively.

3 PIV was found in rats, as in humans. PIV was abolished by neonatal treatment with capsaicin and by administration of CGRP₈₋₃₇ but remained unchanged with SR140333, SR48968 and SR142801 treatments. Prostaglandin inhibition resulted in a significant decrease in PIV. Inhibition of NOS abolished PIV, whereas inhibition of neuronal NOS caused a diminution of PIV.

4 These data suggest that PIV depends on capsaicin-sensitive fibres in rats, as in humans. It appears that CGRP plays a major role in the PIV, whereas neurokinins have no role. Furthermore, PIV involves a contribution from prostaglandins and depends on endothelial NO, whereas neuronal NO has a smaller role.

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Abbreviations: a.u., arbitrary units; CGRP, calcitonin gene-related peptide; DMSO, dimethyl sulphoxide; LDF, laser Doppler flow; L-NNA, N^G-nitro-L-arginine; 7-NI, 7-nitroindazole; NK₁, neurokinin-1; NK₂, neurokinin-2; NK₃, neurokinin-3; NKA, neurokinin A; NKB, neurokinin B; NO, nitric oxide; NOS, nitric oxide synthase; PIV, pressure-induced vasodilation; SP, substance P

Introduction

Over the last two decades it has become clear that cutaneous vasodilation can be caused by the release of mediators from afferent nerve endings and from the endothelium. The cutaneous circulation participates in reflexes of nonthermoregulatory origin (Johnson, 1986; Rowell *et al.*, 1973), such as external pressure stress (Fromy *et al.*, 1998). Capsaicin-sensitive afferent neurones are connected to cutaneous receptors (Holzer, 1991) which enable them to detect noxious stimuli that are potentially or actually harmful to the tissue. Nevertheless, little is known about the response to non-noxious pressure application (Garell *et al.*, 1996; Koltzenburg & Handwerker, 1994).

It is well known that ulcers are easily developed in paralysed patients (Schubert & Fagrell, 1991) and in elderly subjects (Schubert *et al.*, 1994). Risk factors of decubitus ulcers are multiple, but the major ones are the intensity and the duration of applied pressure on the skin, particularly in subjects that remain immobile, and at bony prominences. The skin becomes ischaemic, and if the local pressure is maintained, necrosis occurs. Skin blood flow is regulated to respond to physical stimuli before such a noxious event occurs, in order to prevent ischaemia in the tissue. We

recently reported a significant transient increase of cutaneous laser Doppler flow (LDF) during local non-noxious pressure applied progressively in the skin of humans (Fromy *et al.*, 1998). A similar phenomenon was reported by Schubert & Fagrell (1989) in humans, during moderate step local pressure increases over the sacrum and in the gluteus region. In our previous study (Fromy *et al.*, 1998), we found that capsaicin desensitization resulted in the total disappearance of this local pressure-induced vasodilation (PIV). These results demonstrate the existence of a vasodilatory reflex response to non-noxious pressure strain which is initiated by capsaicin-sensitive nerve terminals in human skin. This local reflex may have important implications for the cutaneous pathologies involved in various neurological diseases and in the pathophysiology of decubitus ulcers (Schubert & Fagrell, 1989).

In the present study, our first aim was to develop an animal model of the PIV to facilitate the study of mediators involved in this reflex. For this purpose, we verified the existence of a PIV in the skin of untreated anaesthetized rats. The involvement of capsaicin-sensitive fibres in this mechanism was then tested in rats treated neonatally with capsaicin. The properties peculiar to capsaicin and its characterization as a tool for sensory neurone research have been demonstrated (Jancso *et al.*, 1967; 1968). Systemic treatment with capsaicin, as a denervating agent in neonatal rats, provokes

*Author for correspondence;
E-mail: jeanlouis.saumet@univ-angers.fr

the most extensive and consistent degeneration of capsaicin-sensitive fibres (Jancso *et al.*, 1977; Saumet & Duclaux, 1982).

Our second aim was to verify whether similar neuropeptide receptors are involved in PIV as those which are involved in responses induced by nociceptive stimuli. Calcitonin gene-related peptide (CGRP) and neurokinins, among the main sensory neuropeptides, are released from small afferent nerve endings following their activation (Maggi & Meli, 1988; Otsuka & Yoshioka, 1993; Rossi & Johansson, 1998). It is known that neuropeptides (CGRP and neurokinins) are expressed, co-stored and co-released from capsaicin-sensitive primary afferent neurones. These concepts have received extensive confirmation over the past several years (Holzer, 1988; Maggi & Meli, 1988). Specifically, CGRP, a potent vasodilator (Brain *et al.*, 1985; Kawasaki *et al.*, 1988), has been co-localized with substance P (SP) in subepidermal and epidermal C-fibres (Gibbins *et al.*, 1985). It can be selectively antagonized by CGRP_{8–37} (Chiba *et al.*, 1989; Donoso *et al.*, 1990; Hughes & Brain, 1991). Thus it is likely that CGRP and/or neurokinin receptors are involved in the mechanism of PIV. The neurokinin-1 (NK₁) receptor is operationally defined as the mediator of the biological activities for which SP is a more potent agonist than neurokinin A (NKA) or neurokinin B (NKB) (Maggi, 1995; Scholzen *et al.*, 1998). This definition does not imply that SP is the exclusive activator of NK₁ receptor in physiological conditions (Otsuka & Yoshioka, 1993; Yanagisawa & Otsuka, 1990). SR140333 is a selective antagonist of NK₁ receptors in various animal species (Emonds-Alt *et al.*, 1993a; Herbert & Bernat, 1996; Jung *et al.*, 1994). The neurokinin-2 (NK₂) receptor presents the following rank order of potency of natural tachykinins: NKA > NKB > SP (Emonds-Alt *et al.*, 1992; 1993b; Maggi *et al.*, 1993). SR48968 is a selective non-peptide antagonist of the NK₂ receptor (Advenier *et al.*, 1992; Emonds-Alt *et al.*, 1992; 1993b; Maggi *et al.*, 1993) and is a useful tool for studying the distribution and function of NK₂ receptors (Emonds-Alt *et al.*, 1993b; Maggi *et al.*, 1993). The neurokinin-3 (NK₃) receptor is defined by the following rank order of potency of natural tachykinins: NKB > NKA > SP (Maggi *et al.*, 1993). SR142801 is a selective non-peptide antagonist of the NK₃ receptor (Emonds-Alt *et al.*, 1995) and is a tool for investigation of the physiological and pathological role of the NK₃ receptor.

The last aim of the present study was to examine whether the mechanism for the PIV response is endothelium dependent and to elucidate the roles of potential factors involved. Both prostaglandins and nitric oxide (NO) are possible mediators of an endothelial vasodilator mechanism (Shastry *et al.*, 1998; Warren *et al.*, 1994). The importance of prostaglandins in the microcirculation has been emphasized by cyclo-oxygenase inhibition, which attenuates the microvascular vasodilator response to inflammation (Williams & Peck, 1977). The role of prostaglandins was studied *via* administration of indomethacin (Friese *et al.*, 1997; Whittle *et al.*, 1980).

NO mediates most, if not all, the functions of endothelium derived relaxing factor (Furchgott & Zawadzki, 1980; Griendling & Alexander, 1996; Moncada *et al.*, 1991; Palmer *et al.*, 1987). NO was identified as a vasodilating substance that is synthesized from L-arginine (Moncada *et al.*, 1989; Moncada & Higgs, 1993) by nitric oxide synthase (NOS) in the vascular endothelium. NO can also be released by autonomic nerves and may contribute to neurally mediated vasodilation in various tissues (Rajfer *et al.*, 1992; Taylor & Bishop, 1993; Toda & Okamura, 1991). Indeed, two constitutive NO synthases have been described in vessels, an endothelial and a neuronal NOS. Both of these NOS isoforms can be blocked

using N^G-nitro-L-arginine (L-NNA) (Vargas *et al.*, 1991). Recently 7-nitroindazole (7-NI), a specific inhibitor of neuronal NO synthase *in vitro*, has been identified (Babbedge *et al.*, 1993; Moore *et al.*, 1993), allowing the differentiation between neuronal and endothelial NOS activities.

Thus, in the present study, we sought to verify the presence of PIV in the skin of anaesthetized rats, and then to elucidate some of the mechanisms for this response. In all, eight related questions were addressed: (1) Does PIV exist in rats, as in humans? (2) Are capsaicin-sensitive fibres involved in PIV in rats? (3) Does CGRP play a major role in PIV? Are substance P (4), or other neurokinins (5) essential for the development of PIV? (6) Does the prostaglandin pathway play a major role in PIV? (7) Is the NO pathway essential for the development of PIV? (8) If so, is neuronal NO involved in this vasodilator response?

Methods

Animal instrumentation

The studies were performed in 218 Wistar rats (250–350 g). At least 2 days prior to the experiment (to prevent skin irritation during the experiment from confounding the results), the hair was removed from the heads of the animals with a depilator lotion, to present a hairless area for the LDF measurements and local pressure application. Animals were housed in a regulated environment with a constant ambient temperature of 24°C. Animals were deprived of food for 24 h prior to the experiments but allowed free access to tap water. Procedures for the maintenance and use of the experimental animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85–23, revised 1985).

For the experiments, animals were anaesthetized by intraperitoneal injection of thiopental at a dose of 50 mg kg^{−1} body weight. The level of anaesthesia was determined by testing eye reflexes. The left femoral vein was cannulated for drug or vehicle administration if necessary. In protocol 1, an arterial catheter was placed in the femoral artery to measure the mean arterial blood pressure of the rats. Rats were placed in an incubator (MMS, Chelles, France) warmed to 30°C to maintain stable body temperature, which was monitored with a rectal thermometer. The animals were placed in the prone position and the head was fixed on a frame. The rats were separated randomly into 16 groups, required for eight protocols.

Experimental procedure for assessment of PIV

A weighbridge was adapted to hold a laser Doppler probe (PF408, Periflux, Perimed, Sweden). The probe has a 12.6 mm² circular contact surface and the measurement was performed in the centre of the hairless area. The probe is connected to a laser Doppler flowmeter (PF4001 Master, Periflux, Perimed, Sweden), which provides a good indicator of the response pattern of skin blood flow from the region of illuminated skin (Saumet *et al.*, 1986). It has previously been shown that LDF measured from the skin surface is not influenced by blood flow to underlying skeletal muscle (Saumet *et al.*, 1988).

Externally applied pressure was increased progressively at 11.1 Pa s^{−1} (5 mmHg min^{−1}) through the LDF probe. The technical details of this method have been described (Fromy *et al.*, 2000). Briefly, the weighbridge was carefully equili-

brated and then positioned in the middle of the hairless skull of the rat. The LDF signal was digitized with a 5 Hz sample frequency using a computerized acquisition system (Biopac, Santa Barbara, California, U.S.A.). Data collection began with a 2-min control period prior to the onset of increasing pressure. The signals were continuously recorded for 30 min. The LDF signals were averaged every 30 s to reduce the instantaneous variability of the signals due to vasomotion. Rectal temperature was recorded throughout the experiment to verify the thermal stability of the rat. All animals were sacrificed at the end of the experiment by an overdose of the anaesthetic agent.

Protocol 1: PIV existence in rats In protocol 1, the existence of PIV in untreated anaesthetized rats ($n=12$) was verified. We monitored systemic haemodynamics *via* the measurement of mean arterial blood pressure. Cutaneous vascular resistance was calculated as the ratio of mean arterial blood pressure to LDF, and thus had the units mmHg a.u.⁻¹.

Protocol 2: Capsaicin-sensitive fibre involvement in PIV To investigate the potential involvement of capsaicin-sensitive fibres in PIV the protocol was performed in rats treated neonatally with capsaicin ($n=22$). A separate group of untreated rats ($n=17$) was used as control.

Capsaicin was injected in neonatal rats intraperitoneally once a day for 5 days (50 mg kg⁻¹). This treatment has been shown to destroy permanently the majority of small unmyelinated sensory nerve fibres (Jancso *et al.*, 1977) including those implicated in nociception. We tested the nociceptive threshold in 8–10-week-old rats by a tail-flick test to verify the efficacy of the treatment. Capsaicin-treated neonatal rats showed a significant increase in the latency (from 15.6 ± 0.8 s for untreated rats to 36.4 ± 1.3 s for treated rats, $P < 0.001$) of the response to the noxious thermal stimulus in the tail-flick test.

Protocol 3: CGRP receptor involvement in PIV To examine the role of the CGRP receptor in this vasodilator response, we used the CGRP antagonist (CGRP_{8–37}) which was injected intravenously (100 µg kg⁻¹) 10 min prior to the start of the experiment ($n=17$). A control group ($n=15$) received the same volume of vehicle as the treated rats.

Protocol 4: NK₁ receptor involvement in PIV To test whether the NK₁ receptor was involved in the vasodilator response, we used a NK₁ receptor antagonist (SR140333), which was injected intravenously (200 µg kg⁻¹) 5 min prior to the start of the experiment ($n=20$). A control group ($n=17$) received intravenously the same volume of vehicle as the treated rats.

Protocol 5: NK₂ and NK₃ receptors involvement in PIV To test whether the NK₂ and/or NK₃ receptors were involved in the vasodilator response, we used NK₂ and NK₃ receptor antagonists. The NK₂ receptor antagonist (SR48968) was injected into the femoral vein as a bolus (4 mg kg⁻¹) 20 min prior to the start of the experiment ($n=16$). The NK₃ receptor antagonist (SR142801) was injected intravenously (1 mg kg⁻¹) 20 min prior to the start of the experiment ($n=15$). A control group ($n=13$) received intravenously the same volume of vehicle as treated rats and were studied 20 min after the injection.

Protocol 6: Prostaglandin involvement in PIV Indomethacin was injected intraperitoneally (5 mg kg⁻¹) 30 min prior to the

start of the experiment ($n=9$). Inhibition of cyclo-oxygenase allowed us to test the role of prostaglandins in the PIV response. A control group ($n=9$) received the same volume of saline as the treated rats intraperitoneally.

Protocol 7: NO involvement in PIV L-NNA was injected in the femoral vein as intravenous bolus (20 mg kg⁻¹) 15 min prior to the start of the experiment ($n=9$). A control group ($n=9$) received the same volume of saline as the treated rats intravenously and were studied 15 min after the saline injection.

Protocol 8: Neuronal NO involvement in PIV A single dose (50 mg kg⁻¹) of 7-NI was injected intraperitoneally 45 min prior to starting the recordings ($n=9$). The control animals ($n=9$) were treated with the same volume of vehicle, and the recordings were started 45 min after its administration.

Drugs

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) was purchased from Sigma company (St. Louis, Missouri, U.S.A.) and was dissolved in 10% ethanol, 10% Tween 80 and 80% distilled water. CGRP_{8–37}, purchased from Sigma company (St. Louis, Missouri, U.S.A.), was dissolved in saline. SR140333 ((S)-1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2.2.2]octane chloride) (lot no. PG 13 2566), SR48968 ((S)-(N)-methyl-N[4-(acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide) (lot n°GX 4 677), SR142801 ((S)-(N)-{1-[3-(benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl]-4-phenylpiperidin-4-yl}-N-methylacetamide) (lot n°VG 15 815) were kindly provided by Dr Emonds-Alt (Sanofi Recherche, Montpellier, France). SR140333 was dissolved in 4% ethanol and 96% saline. SR48968 and SR142801 were dissolved in 10% dimethyl sulphoxide (DMSO) and 90% saline. Indomethacin (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid) and L-NNA were purchased from Sigma company (St. Louis, Missouri, U.S.A.) and were dissolved in saline. 7-NI, purchased from Sigma company (St. Louis, Missouri, U.S.A.), was dissolved in a mixture of 50% Tween 80 and 50% saline.

Analyses of results

Results are expressed as mean $\pm$ s.e.mean. Resting values were calculated as the average over the 2 min of the control period prior to the local pressure application.

To determine the significance of our results between groups in every protocol, we performed a paired *t*-test or one-way ANOVA with repeated measures with a Bonferroni post test. In figures, following confirmation that two groups had overall responses that were significantly different, mean LDF for individual values of applied pressure were compared by *t*-test. One-way ANOVA with Dunnett's multiple comparison test (baseline as control) was performed to determine the differences due to the locally applied pressure within each group. A two-tailed *P* value less than 0.05 was regarded as statistically significant.

Results

Rectal temperature did not change significantly in any experiment. Further, for each protocol, baseline LDF values and the LDF values measured at the maximal applied

pressure were not significantly different between the drug-treated rats and the vehicle treated rats.

Protocol 1: PIV existence in rats

Mean LDF, mean arterial blood pressure, mean vascular resistance and mean rectal temperature with increasing pressure for untreated rats are presented in Figure 1. As can be seen in this figure, mean arterial blood pressure and rectal temperature were stable throughout the experiment. Mean resting LDF was 98.9 ± 4.8 arbitrary units (a.u.) in

untreated rats. A significant increase in mean LDF was found from 0.7 kPa (117.7 ± 7.7 a.u., $P < 0.01$) and reached a maximal mean value of 124.0 ± 8.5 a.u. at 1.3 kPa. Over the same range of pressures, mean vascular resistance decreased from 1.20 ± 0.09 mmHg a.u.⁻¹ to 0.98 ± 0.09 mmHg a.u.⁻¹ ($P < 0.0001$). With further increases in pressure, mean LDF decreased slowly and reached 103.7 ± 9.9 a.u. at 3.3 kPa. Mean values were significantly lower than mean resting LDF at 5.7 kPa (82.2 ± 9.1 a.u., $P < 0.05$). Mean LDF continued to decrease down to a plateau reached at the end of the experiment (46.1 ± 4.0 a.u.).

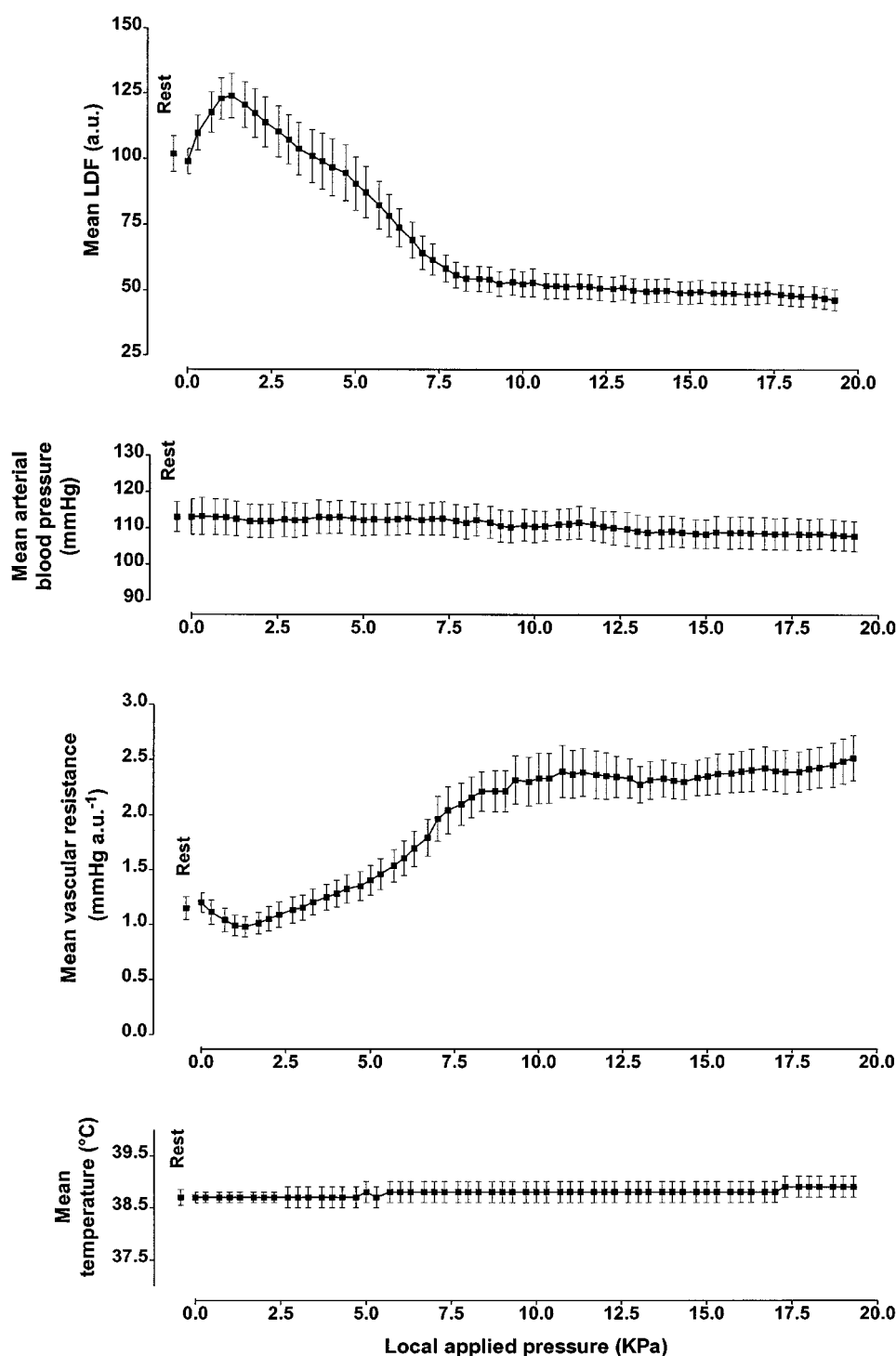


Figure 1 LDF, arterial blood pressure, vascular resistance and rectal temperature (mean $\pm$ s.e.mean) obtained with 11.1 Pa s^{-1} local external pressure application in untreated anaesthetized rats. The first point, outside the scale of pressure, corresponds to the mean resting value before the start of local applied pressure.

Protocol 2: Capsaicin-sensitive fibre involvement in PIV

Mean LDF calculated with increasing pressure for untreated rats and rats treated with capsaicin are presented in Figure 2. Comparison between groups showed a significant difference ($P < 0.001$).

The rats treated with capsaicin had a mean resting LDF of 123.9 ± 8.1 a.u. These rats did not show a significant increase in mean LDF at any time during the experiment. Mean LDF decreased to 119.6 ± 7.3 at 1.7 kPa, and decreased significantly to 107.5 ± 5.9 a.u. at 3.3 kPa ($P < 0.05$). With further pressure increases, mean LDF continued to decrease until it reached a plateau at the end of the experiment (57.3 ± 4.2 a.u.).

Mean resting LDF was 115.1 ± 6.2 a.u. in untreated rats. A significant increase in mean LDF was found from 0.3 kPa (117.4 ± 6.3 a.u., $P < 0.01$) and reached a maximal mean value of 153.3 ± 9.2 a.u. at 2.7 kPa. With further increases in pressure, mean LDF decreased slowly, and was decreased to 150.4 ± 8.6 a.u. at 3.3 kPa. Mean LDF reached 101.6 ± 6.7 a.u., a value significantly lower than mean resting LDF, at 6.3 kPa ($P < 0.05$) and continued to decrease down to a plateau reached at the end of the experiment (51.1 ± 4.3 a.u.).

Protocol 3: CGRP receptor involvement in PIV

Mean LDF measured with increasing pressure in rats treated with CGRP₈₋₃₇ or with its vehicle are presented in Figure 3. The comparison between groups showed a significant difference ($P < 0.001$).

In this set of experiments, rats were treated with CGRP₈₋₃₇ ($100 \mu\text{g kg}^{-1}$, i.v.). The rats had a mean resting LDF of 110.1 ± 6.8 a.u. and did not show significant increase in mean LDF at any time during the experiment. As soon as the applied pressure increased, a continuous decrease of cutaneous blood flow began, reaching 109.2 ± 6.8 a.u. at 0.3 kPa. The observed decrease became significant at 1.7 kPa (105.0 ± 6.6 a.u., $P < 0.05$). With further pressure increases, mean LDF continued to decrease until it reached a plateau at the end of the experiment (60.1 ± 4.4 a.u.).

The rats that received the vehicle of CGRP₈₋₃₇ had a mean resting LDF of 105.7 ± 4.8 a.u. and showed a significant increase of mean LDF at 0.3 kPa (112.4 ± 5.6 a.u., $P < 0.01$).

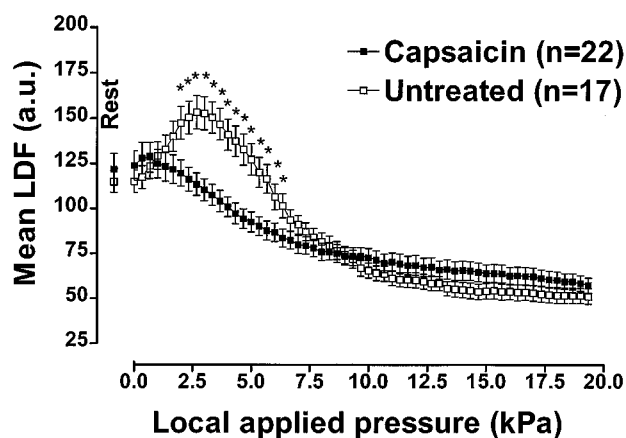


Figure 2 Capsaicin-sensitive fibre involvement in PIV. Mean $\pm$ s.e.mean of LDF obtained with 11.1 Pa s^{-1} local external pressure application in rats treated with capsaicin as neonates and in untreated rats. The first point, outside the scale of pressure, corresponds to the mean resting value before the start of local applied pressure. Asterisks denote significant differences from control data ($*P < 0.05$).

The maximal increase in mean LDF was 125.6 ± 6.9 a.u., occurring at 2.0 kPa. Further increases in pressure led to a progressive decrease in mean LDF, which reached significance for 85.9 ± 6.1 a.u. at 6.0 kPa ($P < 0.05$). The plateau, reached at the end of the experiment, was 65.8 ± 4.5 a.u.

Protocol 4: NK₁ receptor involvement in PIV

Comparison between both groups of rats treated with SR140333 or with its vehicle did not show any significant difference.

Mean resting LDF was 103.3 ± 8.3 a.u. in the rats treated with SR140333 ($200 \mu\text{g kg}^{-1}$, i.v.). In this group, mean LDF was significantly increased above the baseline starting at 0.3 kPa (106.6 ± 9.0 a.u., $P < 0.05$) and continued to increase progressively, reaching a maximal mean value of 116.7 ± 8.8 a.u. at 1.7 kPa. Further increases in pressure resulted in a continuous decrease of mean LDF, which was decreased compared with resting values at 6.0 kPa (86.4 ± 7.0 a.u., $P < 0.05$). Mean LDF continued to decrease until it reached a plateau at the end of the experiment (57.5 ± 4.3 a.u.).

The rats that received the vehicle of SR140333 had a mean resting LDF of 106.6 ± 8.5 a.u. In these rats, mean LDF increased to 111.7 ± 9.1 a.u. at 0.3 kPa ($P < 0.05$) and reached a maximal mean value of 122.0 ± 9.2 a.u. at 1.7 kPa. With further pressure increases, mean LDF decreased, reaching significance at 6.0 kPa (92.9 ± 8.8 a.u., $P < 0.05$) and continued to decrease down to a plateau at the end of the experiment (61.4 ± 4.2 a.u.).

Protocol 5: NK₂ and NK₃ receptors involvement in PIV

Comparison between groups of rats treated with SR48968 or SR142801 or with their common vehicle did not show any significant differences. This indicates that as was true for the NK₁ receptor, blockade of NK₂/NK₃ receptor did not affect the transient vasodilator response.

The rats treated with the NK₂ receptor antagonist (SR48968) had a mean resting LDF of 95.0 ± 2.0 a.u. A significant increase in mean LDF occurred starting at 0.3 kPa (97.7 ± 2.1 a.u., $P < 0.01$) which reached a maximal mean value of 105.1 ± 2.2 a.u. at 1.7 kPa. As with previous protocols, further increases in applied pressure resulted in

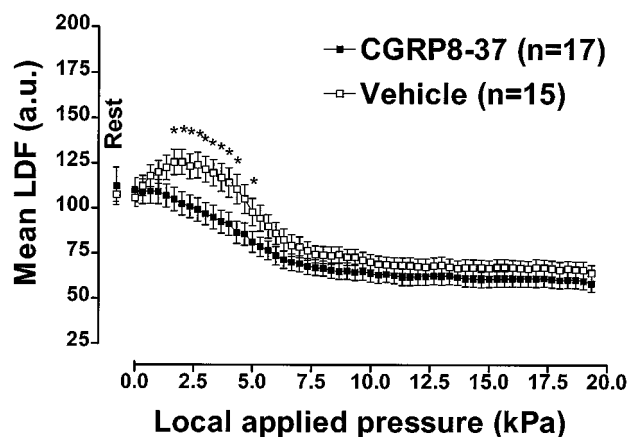


Figure 3 CGRP receptor involvement in PIV. Mean $\pm$ s.e.mean of LDF obtained with 11.1 Pa s^{-1} local external pressure application in rats treated with CGRP₈₋₃₇ ($100 \mu\text{g kg}^{-1}$, i.v.) and with vehicle (saline, i.v.). The first point, outside the scale of pressure, corresponds to the mean resting value before the start of local applied pressure. Asterisks denote significant differences from control data ($*P < 0.05$).

progressive decreases in mean LDF. The observed decrease became significantly different from baseline at 6.0 kPa (82.7 ± 4.4 a.u., $P < 0.05$). With further increases in pressure, mean LDF continued to decrease, until the plateau was attained at the end of the experiment (56.0 ± 2.6 a.u.).

Mean resting LDF was 93.4 ± 2.7 a.u. in the rats treated with the NK₃ receptor antagonist (SR142801). A significant increase of mean LDF was found starting from 0.3 kPa (96.2 ± 3.3 a.u., $P < 0.01$) and reached a maximal mean value of 106.8 ± 3.5 a.u. at 1.7 kPa. Further increases in pressure resulted in a continuous decrease of mean LDF. The plateau, reached at the end of the experiment, was 56.9 ± 1.6 a.u.

The rats that received the vehicle of SR48968 and SR142801 had a mean resting LDF of 98.9 ± 3.0 a.u. A significant increase in mean LDF was found starting from 1.0 kPa (100.7 ± 4.2 a.u., $P < 0.01$) and reached a maximal mean value of 105.3 ± 4.5 a.u. at 2.7 kPa. With further increases in pressure, mean LDF decreased slowly. Mean LDF was significantly lower than mean resting LDF at 6.3 kPa (82.7 ± 4.9 a.u., $P < 0.05$) and continued to decrease until it reached a plateau at the end of the experiment (56.8 ± 1.9 a.u.).

Protocol 6: Prostaglandin involvement in PIV

The mean LDF calculated with increasing pressure in rats treated with indomethacin or with its vehicle are presented in Figure 4. The comparison between groups showed a significant difference ($P < 0.001$), but PIV was not entirely abolished by indomethacin treatment (see Figure 4).

Mean resting LDF in the rats treated with indomethacin was 106.6 ± 6.7 a.u. and increased to 117.1 ± 12.9 a.u. at 1.7 kPa. Further increases in pressure led to a continuous decrease in mean LDF, and decreased to 78.1 ± 7.2 a.u. at 6.3 kPa ($P < 0.05$). The plateau, reached at the end of the experiment, was 54.2 ± 5.6 a.u.

The rats that received the vehicle had a mean resting LDF of 127.0 ± 4.9 a.u. and showed a significant increase starting at 0.7 kPa (155.2 ± 8.4 a.u., $P < 0.05$) and reached a maximal mean value of 167.8 ± 14.2 a.u. at 1.7 kPa. With further pressure increases, LDF continued to decrease to reach significance at 5.0 kPa (98.4 ± 9.0 a.u., $P < 0.05$). The plateau reached at the end of the experiment was 56.9 ± 6.7 a.u.

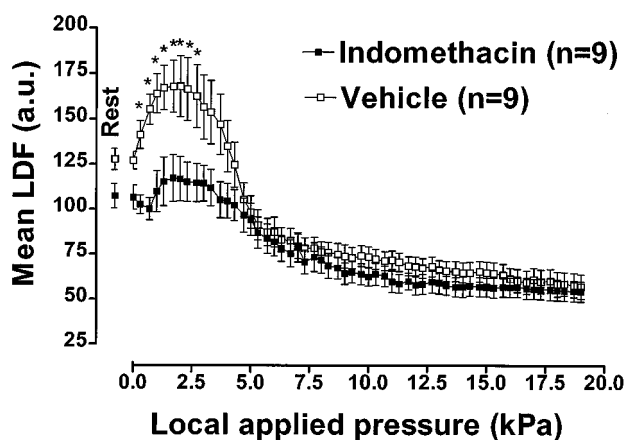


Figure 4 Prostaglandin involvement in PIV. Mean $\pm$ s.e. mean of LDF obtained with 11.1 Pa s^{-1} local external pressure application in rats treated with indomethacin (5 mg kg^{-1} , i.p.) and with vehicle (saline, i.p.). The first point, outside the scale of pressure, corresponds to the mean resting value before the start of locally applied pressure. Asterisks denote significant differences from control data ($*P < 0.05$).

Protocol 7: NO involvement in PIV

Mean LDF values with increasing pressure in rats treated with L-NNA or with its vehicle are presented in Figure 5. The comparison between L-NNA and control groups showed a significant difference ($P < 0.001$).

No vasodilation was observed in L-NNA treated rats. The mean resting LDF was 102.0 ± 12.9 a.u. and mean LDF began to decrease immediately with pressure increase. The decrease was significant at 2.0 kPa (97.2 ± 12.2 a.u., $P < 0.05$) and LDF further decreased with increasing pressure: 94.6 ± 11.9 a.u. at 2.7 kPa and down to 60.9 ± 10.0 a.u. at the end of the experiment.

In the group that received the vehicle, the mean resting LDF was 99.7 ± 8.6 a.u. Mean LDF increased significantly at 2.0 kPa (117.4 ± 14.8 a.u., $P < 0.01$). The maximum increase of mean LDF was 119.8 ± 12.8 a.u. ($P < 0.01$) and occurred at a pressure of 2.7 kPa. With further increasing pressure, mean LDF decreased and became significantly different compared to the mean basal LDF at 9.3 kPa (78.0 ± 4.7 a.u., $P < 0.05$). The plateau, reached at the end of the experiment, was 64.0 ± 4.9 a.u.

Protocol 8: Neuronal NO involvement in PIV

Mean LDF measured with increasing pressure in rats treated with 7-NI or with its vehicle are presented in Figure 6. The comparison between groups showed a significant difference ($P < 0.001$).

In the last set of experiments, rats were treated with 7-NI. The rats had a mean resting LDF of 109.2 ± 7.0 a.u. and showed a significant time averaged increase of LDF starting at 1.3 kPa (144.2 ± 13.0 a.u., $P < 0.05$) which increased to 148.1 ± 11.7 a.u. at 1.7 kPa. Further increases in pressure led to progressive decreases in mean LDF. The decrease reached significance at 6.3 kPa (89.1 ± 5.2 a.u., $P < 0.05$) and continued down to a plateau of 48.0 ± 3.4 a.u. at the end of the experiment.

The vehicle group had a mean resting LDF of 120.8 ± 9.8 a.u. and increased to 179.6 ± 19.6 a.u. at 1.0 kPa ($P < 0.05$). Further increases in pressure led to progressive decreases in mean LDF, which reached significance at 5.0 kPa (96.0 ± 4.6 a.u., $P < 0.05$). The plateau reached at the end of the experiment was 56.9 ± 3.2 a.u.

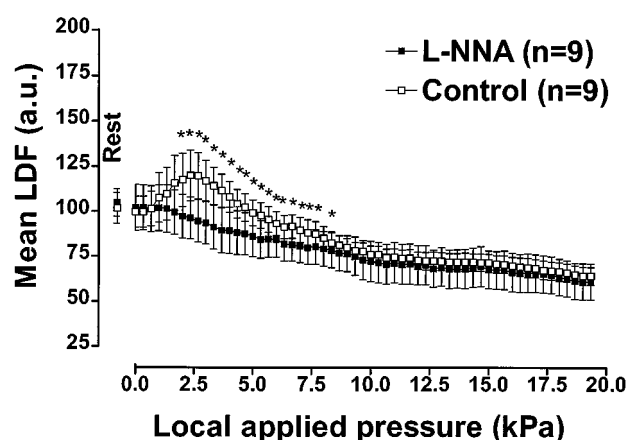


Figure 5 NO involvement in PIV. Mean $\pm$ s.e. mean of LDF obtained with 11.1 Pa s^{-1} local external pressure application in rats treated with L-NNA (20 mg kg^{-1} , i.v.) and with vehicle (saline, i.v.). The first point, outside the scale of pressure, corresponds to the mean resting value before the start of locally applied pressure. Asterisks denote significant differences from control data ($*P < 0.05$).

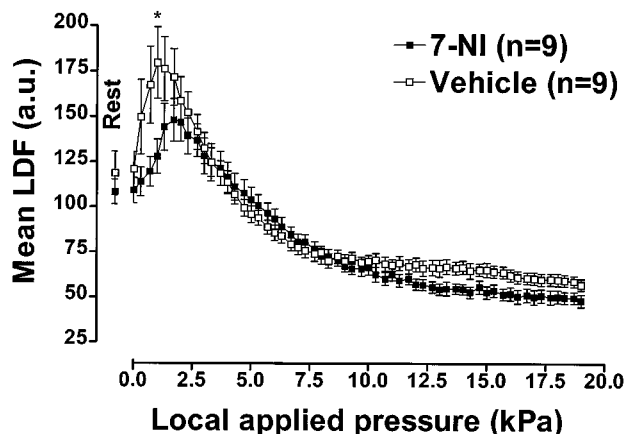


Figure 6 Neuronal NO involvement in PIV. Mean $\pm$ s.e. mean of LDF obtained with 11.1 Pa s^{-1} local external pressure application in rats treated with 7-NI (50 mg kg^{-1} , i.p.) and with vehicle (tween-saline, i.p.). The first point, outside the scale of pressure, corresponds to the mean resting value before the start of locally applied pressure. Asterisk denotes significant differences from control data (* $P < 0.05$).

Discussion

Many investigations have been conducted to examine the thermal vascular responses elicited by cutaneous nociceptors (Handwerker & Kobal, 1993; Lynn, 1994), whereas there has been much less quantitative evaluation of the vascular response to mechanical stimuli. In this latter case, studies have been performed with intense stimuli, such as heavy pressure strains, but little is known about moderate or progressive pressure strains. In the rat, the majority of vasoactive C fibres belong to the cutaneous nociceptor class, some of which show relatively low threshold mechanical sensitivity (Gee *et al.*, 1997; Hu & Sessle, 1988; Lynn *et al.*, 1996).

We report here that progressive external pressure application resulted in a significant transient increase in mean LDF in rat skin, while mean arterial blood pressure remained stable during this increase. This increase in LDF indicates a vasodilator response of the skin, confirmed by the vascular resistance curve shown in Figure 1. Thus, our results indicate that cutaneous pressure-induced vasodilation (PIV) exists in rats, as in humans (Fromy *et al.*, 1998). The results show that the cutaneous PIV is mediated by capsaicin-sensitive fibres and that CGRP is directly involved in this local vasodilator response. These conclusions are drawn from the following observations: PIV disappears in rats treated neonatally with capsaicin and in rats treated with CGRP₈₋₃₇, whereas the response remains unchanged in rats treated with various neurokinin receptors antagonists. These latter results demonstrate that neurokinin receptors are not involved in the mechanism of PIV. Furthermore, we show here that PIV is impaired by indomethacin. Thus, this reflex involves a contribution from prostaglandins. Moreover, PIV is totally abolished with L-NNA treatment whereas 7-NI treatment provokes a diminution of PIV. Despite this diminution of PIV amplitude, a significant skin blood flow increase can be noted during 7-NI treatment. We conclude that PIV is dependent on NO and only partially dependent on neuronal NO.

Systemic administration of high doses of capsaicin has a definitive neurotoxic effect on a population of sensory neurones, the extent of damage depending on the dosage, route of administration, species and age of the animals. The most extensive and consistent lesions are produced by the

systemic treatment of neonatal rats (Jancso *et al.*, 1977; Saumet & Duclaux, 1982). To test whether PIV is dependent on capsaicin-sensitive fibres, as observed in humans on the dorsal aspect of the finger (Fromy *et al.*, 1998), repeated administration of capsaicin in neonatal rats was performed in the present study in order to induce an irreversible destruction of these fibres (Jancso *et al.*, 1977; Nagy *et al.*, 1981). This was confirmed with the tail-flick test as previously demonstrated (Nagy & Van der Kooy, 1983). Since the use of capsaicin in neonates provokes a selective degeneration of small unmyelinated fibres, it is a useful tool for the elucidation of the participation of capsaicin-sensitive fibres in PIV. The marked reduction in this vasodilator response in the animals treated with capsaicin indicates that PIV is mediated by capsaicin-sensitive fibres, as in humans (Fromy *et al.*, 1998). Data in both species support the view that PIV is mediated by the activation of unmyelinated cutaneous afferent nerve terminals.

The total disappearance of PIV in rats treated with CGRP₈₋₃₇ indicates that CGRP participates in this vasodilator response. This antagonist was previously used (Holzer & Jolic, 1994; Kato *et al.*, 1996) at a dose that was similar to that used in the present study ($100 \mu\text{g kg}^{-1}$, i.v.). Our results are in accordance with the data of Merhi *et al.* (1998) who reported that the administration of CGRP₈₋₃₇ before sciatic nerve stimulation resulted in significant inhibition (41%) of blood flux responses. They concluded that the peripheral release of sensory neuropeptides, such as SP and CGRP, initiates a cascade of events causing extravasation of plasma and vasodilation within the local microvasculature, where CGRP is implicated as the major mediator of neurogenic vasodilation.

In general, CGRP is a likely candidate to co-mediate the vasodilation (Brain *et al.*, 1985; Escott & Brain, 1993), particularly in view of the finding that circulating CGRP is largely derived from perivascular capsaicin-sensitive nerve terminals (Zaidi *et al.*, 1985). It appears that CGRP plays a major role in capsaicin-sensitive nerve mediated vasodilation (Holzer, 1988), as shown in the present study for non-nociceptive mechanical stimuli. Indeed CGRP₈₋₃₇ was shown to inhibit completely the increase in cutaneous blood flow induced by capsaicin and also by its more potent analogue, olvanil (Hughes *et al.*, 1992). Also in accordance with the present results, it has been shown that vasodilator responses to activation of sensory C-fibre afferents in the pig nasal mucosa and superficial skin are mainly dependent on CGRP, whereas NK₁ receptor mechanisms seem to be of no or minor importance (Rinder & Lundberg, 1996). Delay-Goyet *et al.* (1992) concluded that CGRP rather than SP is likely to mediate the prolonged vasodilation seen in rat skin upon antidromic stimulation of sensory nerves.

The rats treated with SR140333 ($200 \mu\text{g kg}^{-1}$, i.v.) demonstrated transient increases in LDF in response to locally applied pressure that were similar to those seen in control rats. This indicates that the NK₁ receptor does not play a major role in PIV. Doses of SR140333 lower than $100 \mu\text{g kg}^{-1}$ show effective inhibition of various responses, such as neurogenic inflammation (Amann *et al.*, 1995) or nociceptive transmission following trigeminal ganglion stimulation (Michaud *et al.*, 1998). It has been shown that the inhibition is dose-dependent (Herbert & Bernat, 1996). A minimal administration of $100 \mu\text{g kg}^{-1}$, i.v., was needed to attain 100% inhibition of plasma extravasation in several studies (Emonds-Alt *et al.*, 1993a; Herbert & Bernat, 1996), which was still effective 24 h after treatment (Amann *et al.*, 1995; Emonds-Alt *et al.*, 1993a). In the present study, a

treatment regime with $200 \mu\text{g kg}^{-1}$, i.v., of SR140333 was used to ensure effective and selective blockade of NK₁ receptors. The NK₁ receptor is involved in nociceptive neurotransmission and SR140333 represents a potent drug for the relief of pain in humans (Li & Zhao, 1998; Michaud *et al.*, 1998). In contrast, in conscious rats, systemic administration of SR140333 ($100 \mu\text{g kg}^{-1}$, i.v.), at doses which cause inhibition of neurogenic inflammation, has no detectable effect on acute chemo- or thermosensation; although the decrease in thermal nociceptive threshold has been shown to be entirely dependent on capsaicin-sensitive afferents (Amann *et al.*, 1995). Consistent with Amann *et al.* (1995), we found that SR140333 did not modify PIV, suggesting that the response to non-nociceptive mechanical stimulation does not involve NK₁ receptor.

We further pursued the study of neurokinin involvement in PIV with rats treated with the NK₂ and NK₃ receptor antagonists, SR48968 and SR142801, respectively. The rats treated with SR48968 (4 mg kg^{-1} , i.v.) or SR142801 (1 mg kg^{-1} , i.v.) demonstrated a transient increase in skin blood flow in response to locally applied pressure, that was similar to the vasodilator response in control rats, showing that neither the NK₂ nor the NK₃ receptor play a major role in PIV. Santucci *et al.* (1993) studied the effects of SR48968 on the responses evoked by thermal or mechanical nociceptive cutaneous stimulation. Intravenous injection of SR48968 did not inhibit mechanically evoked responses in doses up to 2 mg kg^{-1} , although these doses completely blocked the thermally evoked response. Thus we chose a dose superior at 2 mg kg^{-1} for use in the present study. Fleetwood-Walker *et al.* (1990) demonstrated that in cats, antagonists of NK₂ receptors were found to attenuate cellular responses to thermal but not to mechanical nociceptive stimulation. Although our protocol did not involve nociceptive stimulation, our results are consistent with the report that the NK₂ receptor was not involved in the vasodilator response to mechanical stimulation.

It is known that the NK₃ receptor mediates potent endothelium-dependent vascular relaxation (Mizuta *et al.*, 1995; Patacchini *et al.*, 1995), but few studies have been conducted to investigate its role in the control of the peripheral vasculature. Mizuta *et al.* (1995) demonstrated that SR142801 inhibits NK₃ receptor-induced relaxation in rat mesenteric artery and Patacchini *et al.* (1995) showed an insurmountable antagonism of the NK₃ receptor in the portal vein of the rat. In contrast, we showed in the present study that the NK₃ receptor was not involved in the vasodilator response to non-nociceptive mechanical stimulation.

Thus, the results of the present study show the involvement of CGRP but not of NK₁, NK₂ and NK₃ receptors in cutaneous PIV. These results are consistent with those of Häbler *et al.* (1999), who concluded that in rat glabrous skin the vasodilatation evoked by a low level of activity in small diameter primary afferents resulted from the synergistic action of neurokinins (SP and/or NKA) and CGRP, while in hairy skin neurokinins are involved to a minor extent only. Similarly, multiple evidences arise from literature that neurokinins are unlikely to play a role in vasodilation whereas CGRP appears to be very important in this respect (Holzer, 1991; Lundberg, 1996; Rinder & Lundberg, 1996).

The last goal of the present study was to test whether the efferent pathway of PIV is dependent upon endothelial factors. Both a direct effect on smooth muscle relaxation and an endothelium dependent vasodilation can be hypothesized. In the present work, as in other studies on the neuronal control of microcirculation (Kajekar *et al.*, 1995; Koller *et al.*,

et al., 1998; Shastry *et al.*, 1998), we tested whether prostaglandins and NO are involved in PIV, and specifically, whether this NO is of neuronal origin (Holzer *et al.*, 1995).

Indomethacin is widely used at doses up to 5 mg kg^{-1} in order to test the role of prostaglandins in different processes and organs (Fabricio *et al.*, 1998; Kato *et al.*, 1997; Meadow *et al.*, 1994; Tamori *et al.*, 1998). In protocol 6 of the present study, cyclo-oxygenase inhibition via indomethacin (5 mg kg^{-1}) altered PIV. Holzer *et al.* (1995) reported that indomethacin depressed sodium nitroprusside-induced hyperemia by 65% without altering baseline blood flow in rat skin. These authors suggested that the neurogenic hyperemia induced by administration of NO donors involves the formation of prostaglandins which in turn cause release of the vasodilator calcitonin gene-related peptide (CGRP) from perivascular afferent nerve fibres. Taken together with the results of the present study, this implies that prostaglandins may act as a co-mediator of PIV. Prostaglandins may also have an important role in mediating bradykinin-induced hyperalgesia to mechanical stimulation. Rueff & Dray (1993) showed that certain prostaglandins, as well as bradykinin and cyclic AMP enhance the responsiveness of peripheral sensory neurones to exogenous noxious and non-noxious stimuli.

On the basis of these observations, we also hypothesized that NO, implicated in the regulation of various vascular functions (Moncada *et al.*, 1991), may be responsible for neurogenic vasodilation in skin during PIV in rats. NO is synthesized from L-arginine by NO-synthase (NOS). Two types of constitutive NOS have been identified, endothelial

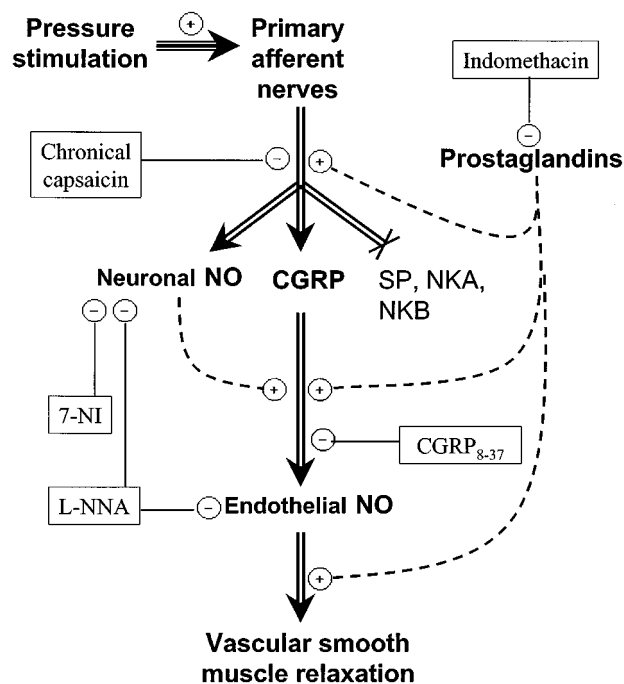


Figure 7 Schematic representation of the suggested mechanisms of vasodilation in response to local applied pressure. Our results show that the local pressure stimulus leads to a release of CGRP and neuronal NO by the primary afferent nerves. CGRP then induces the release of NO by the endothelium and subsequent relaxation of vascular smooth muscle. The results suggest that prostaglandins are involved in PIV. They may act at different levels. Neuronal NO participates to this reflex probably through the release of endothelial NO due to CGRP. Neurokinins do not appear to have any role in this reflex. The level at which each antagonist functions is indicated. Antagonists are represented in oval, negative signs represent inhibitory effects and positive signs represent stimulatory effects on the pathway.

and neuronal (Bredt & Snyder, 1990). Non-specific inhibition of NOS with L-NNA allows for the study of involvement of NO in vasodilation (Preckel *et al.*, 1996; Tabrizi-Fard & Fung, 1996; Taylor & Bishop, 1993). The intravenous administration of L-NNA at a dose of 20 mg kg⁻¹ presents a mean residence time (biological average half-lives of 12 min) in plasma that suited in our experiment (Tabrizi-Fard & Fung, 1996). The total abolition of PIV following L-NNA administration in protocol 7 of the present study leads us to conclude that the NO pathway is essential for PIV. Protocol 8 was performed using 7-NI to determine whether NO release during PIV resulted from the neuronal NOS activation. Kajekar *et al.* (1995) showed a dose dependent inhibition with a significant effect on rat paw skin oedema being observed at a systemic dose of 10 mg kg⁻¹. Although the dose we used (50 mg kg⁻¹) was five times higher than theirs, PIV was diminished, but not totally abolished following 7-NI administration. This strongly suggests that neuronal NOS is partially involved in PIV in rats. Overall, our results are consistent with the idea that NO plays a major role in PIV.

These latter results are consistent with previous evidences which show that both prostaglandins and NO are co-released following stimulation by acetylcholine, ATP or bradykinins (Warren *et al.*, 1994). Our data are also in accordance with Mehri *et al.* (1998) who concluded that NO (among other

mediators) participates in neurogenic vasodilation in rat skin microvasculature.

In summary, we report here that cutaneous pressure-induced vasodilation (PIV) is a phenomenon mediated by capsaicin-sensitive fibres in rats, as it is in humans. PIV was completely inhibited following CGRP antagonism, whereas the response remained unchanged following NK₁, NK₂ or NK₃ receptor antagonism. Moreover, the total disappearance of cutaneous PIV was observed when the non specific NOS inhibitor was administered, whereas inhibition of prostaglandins or specific neuronal NOS only alters the normal response. Our conclusions regarding the mechanisms of cutaneous PIV are summarized schematically in Figure 7. Our data indicate that CGRP receptor plays a major role, whereas the neurokinin receptors do not have a role in the cutaneous vasodilator response to local externally applied pressure. PIV involves a contribution from prostaglandins and is dependent on endothelial NO, whereas neuronal release of NO is only partially involved.

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