Effect of Morphine on Changes in Cutaneous Blood Flow Induced by Antidromic Stimulation of Primary Afferent Fibers in the Hind Instep of Rats

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ABSTRACT—The effects of morphine on the release of immunoreactive substance P (iSP) into the subcutaneous perfusate and the changes in cutaneous blood flow (CBF) elicited by antidromic stimulation of sectioned sciatic nerve were investigated in the instep of the hind paw of rats. Antidromic stimulation of the sectioned sciatic nerve induced a marked increase in iSP release into the subcutaneous perfusate and a biphasic flow response consisting of an initial transient decrease followed by an increase. Both the iSP release and the increase of the CBF evoked by antidromic stimulation (the second phase) were significantly inhibited by intra-arterial (i.a.) infusion of morphine (30 μmol/kg). These inhibitory effects of morphine were antagonized by pretreatment with naloxone (2 mg/kg, i.p.). The i.a. infusion of SP (0.25 pmol/kg) induced a biphasic flow response similar to that elicited by antidromic stimulation of the sectioned sciatic nerve. Neither phase induced by i.a. infusion of SP was affected by preinfusion of morphine (10 or 30 μmol/kg, i.a.). We suggest that morphine applied locally mainly acts on the peripheral endings of small-diameter afferent fibers, not on blood vessels, and that activation of this site is involved in the regulation of the microcirculatory hemodynamics of cutaneous tissue through inhibition of SP release.

Keywords: Substance P, Cutaneous blood flow, Antidromic stimulation, Morphine, Naloxone

Small-diameter primary afferent neurons are known to transmit nociceptive messages to central neurons (1) and also to be important in the inflammatory process in the periphery through axon-reflex mechanisms (2). In fact, vasodilatation, swelling and pain, which are the principal signs of inflammation, can all be produced by the activation of small-diameter primary afferent neurons (3). Several lines of evidence indicate that substances released from capsaicin-sensitive, small-diameter primary afferent neurons mediate these signs of inflammation (4). There is some evidence indicating that substance P (SP) may be one of the predominant mediators responsible for the initiation of neurogenic inflammatory responses. For example, electrical stimulation of small-diameter afferent fibers was shown to induce the release of SP in the periphery (5–7) and to produce the same cutaneous vasodilatation and plasma extravasation as that induced by intradermal or intra-arterial administration of SP (8). These findings suggest that afferent impulses from nerve terminals of small-diameter afferent fibers generated by noxious stimuli may cause the release of SP, which may finally control the inflammatory response through regulation of the microcirculation.

Opioids, which are known to inhibit the release of SP from the central terminals of small-diameter afferent fibers (9, 10), have been also shown to inhibit stimulus-evoked SP release into the subcutaneous space (11) and neurogenic inflammation (12, 13).

The aim of this study was to elucidate the site of action of opioids in peripheral sensory nerves–cutaneous blood vessel systems involved in neurogenic inflammation. For this, we examined the effect of morphine as a representative opioid on the change in cutaneous blood flow (CBF) and SP release into the subcutaneous (s.c.) space evoked by antidromic stimulation of sectioned sciatic nerve in the instep of the hind paw of rats by using the non-invasive technique of laser Doppler flowmetry and the double coaxial perfusion technique, respectively.
MATERIALS AND METHODS

Measurement of blood flow

Male Sprague-Dawley rats (140–160 g body weight) were anesthetized with urethane (780 mg/kg, i.p.) and then paralyzed by an injection of alcuronium (1 mg/kg, s.c.) with supplementary injections of the same dose when necessary. The trachea was cannulated, and artificial ventilation was carried out during the experiments. The sciatic nerve was cut centrally and placed on bipolar stimulating electrodes insulated with enamel. The blood pressure was monitored continuously through a cannula inserted into the common carotid artery by means of a pressure transducer. CBF in the instep of the hind paw was measured with a laser Doppler flowmeter (ALF 2100; Advance, Tokyo). The laser probe was placed on a site 5 mm from the caput of the os metatarsale tertium to basis, which was depilated with commercial depilatory cream. After these operations, the animals were left for at least 1 hr to avoid any influences of treatments including the effect of the depilatory cream.

The change of blood flow was calculated according to the following formula: \((C - B)/A \times 100\), where \(A\) and \(B\) represent the blood flows just before intra-arterial (i.a.) infusion of drugs or saline and just before electrical stimulation of the sciatic nerve or i.a. infusion of SP, respectively, and \(C\) represents the peaks of blood flows induced following electrical stimulation or i.a. infusion of SP.

Release of SP into the subcutaneous space

Male Sprague-Dawley rats (140–160 g body weight) were anesthetized with urethane (780 mg/kg, i.p.). A double polyethylene tube about 5 cm in length (the inner tube of 0.5 mm diameter was 5 mm longer than the external one of 3 mm diameter) was introduced into the s.c. space of the instep. Perfusion was carried out through this double coaxial tube with a peristaltic pump at a rate of 0.1 ml/min with saline containing the aminopeptidase inhibitor bestatin (3 mg/100 ml) and the dipeptidyl carboxypeptidase inhibitor captopril (0.1 mM). Fractions of 1 ml of perfusate were collected through the outer tube in 10-min periods with a fraction collector and then placed in an ice bath. The fractions were lyophilized, and their SP content was measured by radioimmunoassay by the method described by Yonehara et al. (10). The SP antiserum cross-reacted with neurokinin A (1%) and neurokinin B (0.2%). The detection limit of SP was 0.3 fmol.

Electrical stimulation

The sciatic nerve was sectioned proximally, and the end was placed on bipolar, enamel-insulated, stimulating electrodes under liquid paraffin. The sciatic nerve was stimulated for a period of 30 sec or 10 min with a 10 V square wave (frequency 2 Hz, duration 1 msec).

Drug administration

Morphine and SP were dissolved in saline and administered by the i.a. route. Solutions were continuously infused on the side of measurement, via an arterial cannula inserted into the contralateral common iliac artery. In the experiment of laser Doppler flowmetry, infusion of morphine and SP was carried out for a 5-min period from 10 min before electrical stimulation or infusion of SP and for a 2 min period, respectively, with a microinjection pump (CMA/100; Carnegie Medicine, Stockholm, Sweden) at an infusion rate of 10 \(\mu l/min\). In the perfusion experiment, morphine was infused at a rate of 20 \(\mu l/min\) for a 20-min period from 10 min before electrical stimulation until the end of stimulation. The dead volume of the inserted arterial cannula was 10 \(\mu l\). In both experiments, naloxone (2 mg/kg) was injected intraperitoneally 30 min before electrical stimulation.

Substances used

The following compounds were used: morphine hydrochloride (Sankyo Pharmaceutical Co., Tokyo), naloxone hydrochloride, SP (Peptide Institute, Inc., Minoh), and \([125I]\)TyrBSP (New England Nuclear Co., Boston, MA, USA).

RESULTS

Effect of i.a. infusion of morphine on the increase of the iSP release into s.c. perfusate evoked by antidromic stimulation

The iSP concentration in the perfusate was initially high, but gradually decreased with time to a steady level after 50 min, which was maintained for at least 150 min. The iSP level in the resting state, that is, in three 10-min fractions obtained in a 30-min period 50–80 min after starting perfusion was 1.24±0.1 fmol/10 min (n = 10).

Table 1. Effect of intra-arterial infusion of morphine on release of iSP into the subcutaneous perfusate evoked by antidromic stimulation of the sectioned sciatic nerve

| Treatment     | n | Before stimulation (fmol/10 min) | During stimulation (fmol/10 min) |
|---------------|---|----------------------------------|----------------------------------|
| Saline        | 10 | 1.24±0.1                         | 3.90±0.3                         |
| Morphine      | 8  | 1.32±0.1                         | 2.06±0.2                         |
| Morphine + Naloxone | 8 | 1.39±0.09                        | 3.16±0.4                         |

Morphine (30 \(\mu mol/kg\)) and naloxone (2 mg/kg) were administered by i.a. and i.v. routes, respectively. Values represent means±S.E.M. a, P<0.05 when compared with the value obtained in the saline-treated group and morphine-treated group during antidromic stimulation (Student's unpaired t-test).
Antidromic stimulation of the sectioned sciatic nerve for 10 min caused a marked increase in iSP release (3.90±0.3 fmol/10 min, n=10). The i.a. infusion of morphine (30 μmol/kg) attenuated the increase of the iSP release during antidromic stimulation. The inhibitory effect of morphine was significantly antagonized by pretreatment with naloxone at a dose of 2 mg/kg, i.p. (Table 1).

**Effect of i.a. infusion of morphine on the changes in CBF induced by antidromic stimulation**

As shown in the upper part of Fig. 1, antidromic stimulation of the sectioned sciatic nerve induced a biphasic flow response, an initial transient decrease followed by an increase, with no alteration in the blood pressure. The i.a. infusion of morphine (30 μmol/kg) caused a slight decrease of the basal CBF and the blood pressure immediately after the start of its infusion; and it mainly inhibited the second phase (the phase of increase) of the CBF change induced by antidromic stimulation, its effect being dose-dependent. This inhibitory effect of morphine was significantly antagonized by pretreatment with naloxone at a dose of 2 mg/kg, i.p. (Figs. 1 and 2).

**Effect of i.a. infusion of morphine on the changes in CBF induced by i.a. infusion of SP**

Infusion of SP (0.25 μmol/kg) into the common iliac artery evoked a similar response to that induced by antidromic stimulation of the sectioned sciatic nerve; that is,
the CBF decreased, immediately after the start of infusion of SP, to a minimum within 30 sec, and then increased to a maximum about 1–2 min after the start of infusion. However, unlike antidromic stimulation, after reaching maximum, the CBF decreased under the basal level (Fig. 3). The change in CBF evoked by i.a. infusion of SP was unaffected by pretreatment with morphine (10 or 30 μmol/kg, by i.a. infusion) (Figs. 3 and 4).

**DISCUSSION**

In the present study, antidromic stimulation of the sectioned sciatic nerve produced a marked increase in iSP release into the subcutaneous perfusate, and a biphasic flow response consisting of an initial transient decrease, followed by an increase. The laser Doppler flow meter used in the present study emits the light of a 2 mW He-Ne laser (wavelength 632.8 nm) which penetrates approximately 1 mm into the skin. Thus the changes in blood flow measured occur in the microvasculature. In the skin, small diameter nerve fibers containing SP are found in the dermis and are sometimes observed to be associated with small blood vessels (14). Nerve fibers can also be seen to branch from the dermal fibers into the epithelium. Moreover, it is known that treatment of newborn rats with capsaicin results in selective and permanent degeneration of small-diameter afferent fibers with significant decrease in the content of SP (15). We have reported that pretreatment of newborn rats with capsaicin inhibited both phases of the biphasic flow response evoked by antidromic stimulation of the sectioned sciatic nerve (7). From these findings, it seems likely that stimulation-induced changes in CBF are mediated by SP released from the peripheral endings of capsaicin sensitive small-diameter afferent fibers present in the sciatic nerve. This idea is strongly supported by our observation, shown in Fig. 3, that the changes in CBF evoked by antidromic stimulation were mimicked by infusion of SP into the side of measurement via an arterial cannula inserted into the contralateral common iliac artery.
artery.

In the present study, by the laser Doppler flow meter technique, morphine mainly inhibited the phase of increase of the CBF in the biphasic flow response induced by antidromic stimulation, its effect being dose-dependent and reversible by naloxone. It did not affect the changes in CBF evoked by the i.a. infusion of SP. These findings imply that the site of action of morphine in the peripheral sensory nerve-cutaneous blood vessel system involved in neurogenic inflammation is principally at the peripheral endings of capsaicin-sensitive, small-diameter afferent fibers, not on blood vessels. Opiate binding sites have been demonstrated on sensory neurons, and they are transported in capsaicin-sensitive neurons towards the periphery (16, 17). Furthermore, intense staining of immunoreactive opioid receptors was recently detected in the small-diameter cutaneous nerves of rat hind paw (18). In another experiment by the double coaxial perfusion technique, the increase in ISP release evoked by antidromic stimulation of the sectioned sciatic nerve was shown to be inhibited by i.a. infusion of morphine (Table 1). This result was similar to the previous results obtained by antidromic stimulation of the sciatic and saphenous nerves which contain sensory afferent fibers terminating in the hind instep (6). In view of these facts, our results indicate that morphine interacts with opioid receptors on the peripheral endings of capsaicin-sensitive, small-diameter afferent fibers and reduces antidromic vasodilatation by inhibiting the release of SP from the peripheral endings of these fibers.

Opioid peptides have been demonstrated in the dorsal ganglia of various species such as rabbits and rats using antibodies raised against Leu enkephalin, dynorphin and other peptides related to the preproenkephalin B (prodynorphin) system (19, 20). Furthermore, immune cells have been shown to contain and release opioid peptides in vitro (21, 22), and various types of these cells accumulate at sites of inflammation in vivo (23). Taking all these findings into consideration, it seems likely that the peripheral site of action of opioids on capsaicin-sensitive, small-diameter afferent fibers functions as a prejunctional autoreceptor regulating the release of neuropeptides under certain pathophysiological conditions such as inflammation.

Morphine inhibited the increase of SP release induced by antidromic stimulation, but did not affect the initial phase of reduction of CBF observed on antidromic stimulation or i.a. infusion of SP. If this reduction reflects vasoconstriction, SP may initially induce contraction of smooth muscle by activating SP receptors located on smooth muscle cells or by promoting the release of an endothelium-derived contracting factor such as endothelin through activation of the SP receptor in the endothelium.

In this connection, it is noteworthy that tachykinin receptors were found on cultured vascular smooth muscle cells (24) and endothelial cells (25), and the vasorelaxant response to SP was shown to be mediated through the release of endothelium-derived relaxant factors via specific receptors located on endothelial cells (26, 27). These findings suggest the interesting possibility that two types of SP-sensitive binding sites on cutaneous microvessels (smooth muscle-endothelium) in the instep of the hind paw of rats may contribute to vasoconstriction and vasodilatation. It seems likely that the maximal effect at an SP binding site involved in vasoconstriction can be achieved at a lower agonist concentration than that at an SP binding site involved in vasodilatation, and that the doses of morphine used in the present study did not inhibit antidromic stimulation-induced SP release sufficiently to prevent an initial transient decrease.

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