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# Evidence for the participation of glutamate in reflexes involving afferent, substance P-containing nerve fibres in the rat

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**1** Responses mediated, either peripherally or centrally, by substance P-containing primary afferent C-fibres were investigated in the rat following impairment of axonal transport by colchicine (120 $\mu g\ kg^{-1}$, i.p., daily for 3 days), and after treatment with the tachykinin antagonist SR-140333 (10-100 $\mu g\ kg^{-1}$, i.v.) or the N-methyl-D-aspartate (NMDA) antagonist MK-801 (100 $\mu g\ kg^{-1}$).

**2** Peripheral effects mediated by afferent C-fibres were measured by plasma protein extravasation (Evans blue method), following antidromic stimulation of the sciatic nerve, topical application of mustard oil and, as control, i.v. injection of substance P. SR-140333 (100 $\mu g\ kg^{-1}$) reduced the effects by 86%, 75% and 74%, respectively. Colchicine reduced the effects of the first two stimuli by 31% and 33% and, as expected not the effect of substance P. The increase of paw skin temperature following capsaicin i.v. was inhibited by SR-140333, but not by colchicine. MK-801 had no effect on the plasma protein extravasation following antidromic sciatic nerve stimulation or on the rise of paw skin temperature induced by capsaicin i.v., thus excluding an effect of MK-801 on peripheral terminals of afferent neurones.

**3** Depressor reflexes, which are known to be mediated by capsaicin-sensitive afferent neuones, such as those elicited (A) by a stimulating dose of 30 ng capsaicin i.a., (B) by distension of the ascending colon or (C) by afferent sciatic nerve stimulation were studied. Colchicine significantly reduced depressor reflexes A and B, but had no effect on reflex C. None of the reflexes was affected by SR-140333. MK-801 significantly inhibited all three reflexes.

**4** Capsaicin, injected either i.v. (200 $\mu g\ kg^{-1}$) or into the nucleus caudatus/putamen (i.c., 30 $\mu g$), induced an increase in paw skin temperature and a decrease in colon temperature. The rise in fore paw skin temperature ($\Delta t = 2.3 \pm 0.4°C$) evoked by capsaicin i.v. was almost completeley blocked by SR-140333 (100 $\mu g\ kg^{-1}$, i.v.), but no inhibition was observed with MK-801, indicating that capsaicin had brought about a release of substance P from peripheral nerve terminals. Colchicine did not influence heat dissipation induced by i.v. capsaicin.

**5** When capsaicin was injected i.c., the rise in paw skin temperature in colchicine- and SR-140333-pretreated groups did not differ from that of the control group. MK-801 totally prevented the heat loss reaction to i.c. capsaicin administration. Colchicine did not change the effects of i.v. or i.c. injected capsaicin: this excludes the involvement of a mechanism dependent on axonal transport of neurotransmitters.

**6** The reduction of axonal transport by colchicine reduced plasma extravasation induced by mustard oil and antidromic sciatic nerve stimulation (peripheral functions) and depressor reflexes evoked by i.a. capsaicin and colon distension (central functions). It can be argued that afferent stimulation of the sciatic nerve includes the stimulation of A-fibres, which might be less sensitive to colchicine. SR-140333 was effective only on peripherally mediated responses.

**7** The recent evidence for the concomitant release of glutamate and substance P from central terminals of afferent C-fibres, known to mediate reflexes abolished after capsaicin treatment allows the following conclusions: (a) the inhibition by MK-801 indicates an essential role for glutamate in the central transmission of these reflexes; (b) tachykinin antagonists such as SR-140333 do not affect these responses when administered systemically. Centrally released substance P could be involved in functions of the CNS other than those investigated here unless the access of neurokinin antagonists to their receptors in the CNS is insufficient.

**Keywords:** Afferent C-fibres; colchicine; tachykinin antagonist; SR-140333; N-methyl-D-aspartate antagonist; MK-801

## Introduction

There are several ways by which the function of small diameter afferent (C-)fibres can be inhibited *in vivo*: A large dose of capsaicin ($\geq 30$ mg $kg^{-1}$) inhibits the release of peptide neurotransmitters within minutes (Lembeck & Donnerer, 1981; Griesbacher, 1994) and within two days it causes the depletion of substance P and other peptides (for review see Holzer, 1991). Local application of capsaicin onto a peripheral nerve blocks impulse conduction only in small diameter afferent fibres (Petsche *et al.*, 1983) and inhibits the axonal transport of substance P and somatostatin, but not that of noradrenaline and acetylcholinesterase (Gamse *et al.*, 1982). The capsaicin-induced inhibition of axonal transport is prevented by nerve growth factor (Taylor *et al.*, 1985; Donnerer *et al.*, 1992). The effects of capsaicin are, therefore, specific to small diameter afferent fibres and are not limited to those fibres containing substance P.

Colchicine inhibits fast axonal transport (Dahlström, 1968). In contrast to capsaicin, colchicine also inhibits the transport

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of acetylcholine and noradrenaline (Dahlström *et al.*, 1975) and that of cholinesterase (Jackson & Diamond, 1977). In afferent fibres, 37% of the substance P present is mobile and is transported at a rate of 170 mm $day^{-1}$ (Gamse *et al.*, 1982). This is within the range of the transport rate of acetylcholine or noradrenaline (Dahlström *et al.*, 1975). After systemic treatment of rats with colchicine for three days, the plasma protein extravasation induced in the skin either by antidromic nerve stimulation or by topical application of capsaicin was greatly reduced. Although the action of colchicine is not specific for small diameter afferent nerve fibres, its action in these neurones has been established (Garret *et al.*, 1991; Moussaoui *et al.*, 1993).

The recently developed non-peptide tachykinin receptor inhibitors do not impair the function of afferent fibres *per se*. They block the function of tachykinins when these are either injected or endogenously released from peripheral nerve. The antagonist CP-96,345 completely blocked the fall in blood pressure induced by substance P or by neurokinin A, but not that evoked by calcitonin gene-related peptide (CGRP) or by vasoactive intestinal peptide (VIP). CP-96,345 also inhibited the plasma protein extravasation mediated by substance P released from peripheral terminals following antidromic nerve stimulation (Lembeck *et al.*, 1992). These findings have been confirmed with all other neurokinin$_1$ ($NK_1$) receptor blockers (see Discussion). In contrast to these effects on peripheral nerve terminals, other functions attributable to the release of substance P at central nerve terminals were not affected (Griesbacher *et al.*, 1992), except for few examples (see Discussion). Many autonomic reflexes and neuroendocrine regulation mechanisms are eliminated when capsaicin prevents the functions of small afferent fibres (for reveiw see Lembeck, 1988).

The N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801, has recently been found to inhibit autonomic reflexes induced by afferent nerve stimulation. It inhibited the depressor reflex induced by i.a. capsaicin and the somato-sensory pressor reflex evoked by afferent sciatic nerve stimulation (Donnerer & Amann, 1994). The first of these reflexes is mediated by capsaicin-sensitive afferent fibres, whereas the second is mediated by capsaicin-resistant somatic afferent fibres.

Thermal stimulation is signalled mainly via polymodal nociceptors that respond to heat, chemical and mechanical stimuli. Capsaicin acts on the afferent pathway and on the centre in the preoptic-anterior hypothalamic region where the afferent signals are integrated and translated into heat dissipation mechanisms such as vasodilatation in paws and tail (Jancsó-Gábor *et al.*, 1970; Dib, 1982; Donnerer & Lembeck, 1983; Hajós *et al.*, 1987). Pretreatment with a large dose of capsaicin completely blocks this regulation mechanism (Jancsó-Gábor *et al.*, 1970).

In the present investigation, three experimental models of plasma protein extravasation, three types of depressor reflex mediated by capsaicin-sensitive afferent nerves (see Methods) and the effect of capsaicin on thermoregulation were used. Colchicine acts on axonal transport, SR-140333 blocks, predominantly, peripheral tachykinin receptors, and MK-801 blocks central NMDA receptors.

## Methods

### *Animals and treatments*

Rats, Wistar strain (Dobra Voda, Slovakia) of either sex, weighing 250–350 g, were used. In all experiments, except for the heat dissipation studies (see below), the rats were anaesthetized with sodium pentobarbitone (60 mg $kg^{-1}$, i.p.) and fitted with a tracheal cannula to ensure unobstructed respiration. I.v. injections of substances were made via a catheter inserted into a jugular vein. At the end of each experiment the rats were killed.

Three different treatments were used: (1) Colchicine (120 $\mu g\ kg^{-1}$, i.p. daily for the 3 days before the experiment); control animals received corresponding volumes of a 0.9% NaCl solution (saline, 1 ml $kg^{-1}$ $day^{-1}$, i.p.) for 3 days. (2) SR-140333 was administered in doses of 10, 30 or 100 $\mu g\ kg^{-1}$, i.v., 5 min prior to the experiment. (3) MK-801 (100 $\mu g\ kg^{-1}$, i.v.) was injected i.v. 10 min before the experiment.

### *Plasma protein extravasation*

Extravasation of plasma proteins was determined by the Evans blue leakage technique (Lembeck & Holzer, 1979). In order to remove all of the blood from the circulation, the chest was opened, the apex of the heart was cut off, a cannula was inserted through the left ventricle and fixed in the aorta. Then, two portions, each of 50 ml, of saline (the first portion containing 10 iu $ml^{-1}$ heparin) were infused. Extravasated Evans blue in the skin was extracted by soaking in 3 ml formamide for 48 h at 50°C and determined photometrically at 620 nm. The amount of Evans blue in excess of that extracted from the control paw skin was used to quantify the extravasation of plasma proteins.

### *Peripheral function of C-fibres*

*Antidromic sciatic nerve stimulation*: The sciatic nerve was severed and the distal end placed on bipolar platinum electrodes and covered with paraffin oil. Fifteen minutes later, Evans blue (25 mg $kg^{-1}$, i.v.) was injected. Ten minutes afterwards, the nerve was stimulated for 5 min (5 V, 2 ms, 10 Hz). After the end of the stimulation, the amount of Evans blue extravasated into the paw skin area innervated by the sciatic nerve was determined as described above.

*Mustard oil application*: Five minutes after the rat was injected with Evans blue, (25 mg $kg^{-1}$, i.v.), the dorsal side of one hind paw was painted three times at 5 min intervals with 5% (w/w) mustard oil in liquid paraffin. Paraffin oil was applied to the control paw. Five minutes after the last application, the Evans blue extravasated into skin of the dorsal side of the hind paw was measured.

*Substance P-induced plasma extravasation*: Five minutes after the rat was injected with Evans blue (50 mg $kg^{-1}$, i.v.) substance P (13 $\mu g\ kg^{-1}$, i.v.) was injected. After another 5 min, the Evans blue in the skin from the dorsal side of both hind paws was determined as above.

### *Central function of C-fibres*

*Blood pressure measurement*: Carotid blood pressure was continuously recorded by means of a pressure transducer. The depressor response was designated as the maximal decrease in mean arterial pressure.

*Depressor reflex induced by capsaicin infused i.a:* Infusions of a solution of capsaicin (1 $\mu g\ ml^{-1}$, 60 $\mu l\ min^{-1}$) were made at 10 min intervals into a femoral artery via a cannula placed in the superficial epigastric artery. The total amounts of capsaicin infused were 3, 10, 30 and 100 ng capsaicin (Donnerer *et al.*, 1988).

*Depressor reflex response induced by distension of the colon*: In rats that had been fasted for 12 h, a segment of the ascending colon, about 5 cm long, was ligated at the proximal end. The distal end was connected to three bottles each containing saline with surface levels at 20, 40 and 80 cm above the animal (Lembeck & Skofitsch, 1982). The saline flowing into the gut was warmed to 37°C by a heat exchanger. The ligated segment was replaced *in situ* and the abdomen closed. Intraluminal pressure was rapidly increased by opening the connection to one of the bottles for 10 s. The intraluminal pressure was then reduced to normal by opening an outflow

at body level. Increases in colonic intraluminal pressure (20, then 40 and then 80 $cmH_2O$) were made at 5 min intervals and the size of the resulting depressor reflex responses were measured.

*Depressor reflex response induced by stimulation of afferent sciatic nerve fibres*: The proximal end of the severed sciatic nerve was placed on bipolar platinum electrodes. Afferent fibres were successively stimulated electrically (1 ms, 5 Hz) at different voltages (3, 10, 30 V) at intervals of 5 min. The size of the depressor reflex response after each stimulus applied was evaluated.

### *Heat dissipation*

During both sets of experiments, the animals were kept in a thermostatically controlled cage (45 × 70 × 75 cm) at a constant temperature of 30°C. Thermoprobes were placed 6 cm into the colon and on the plantar side of each fore and hind paw. Colon and paw pad temperatures were measured at intervals of 2 min with an electronic thermometer (Digimed H11S; TTW, Waldkirch, Germany), before and after injection of capsaicin.

In the first set of experiments, the rats were given diazepam (25 mg $kg^{-1}$, i.p.) in order to avoid heat production caused by locomotion. Capsaicin (200 $\mu g\ kg^{-1}$, i.v.) was injected via a cannula previously inserted, under anaesthesia, in a jugular vein (Donnerer & Lembeck, 1983). There was no obvious behavioural response to capsaicin.

In the second set of experiments, capsaicin was infused intracerebrally (i.c.) according to Hajós *et al.* (1988). The animals were first anaesthetized with chloral hydrate (400 mg $kg^{-1}$, i.p.) and then placed in a standard stereotaxic instrument (David Kopf, Tujunga, U.S.A.). A guide cannula was introduced into the Nc. caudatus/putamen, the tip being located according to coordinates of Paxinos & Watson (1986): lateral +3.0 mm, anterior +0.5 mm, ventral −5.5 mm, from the bregma. At the end of the experiment, the injection site was marked with Evans blue and the site of the injection checked *post mortem*. The capsaicin infusions were started as soon as the rectal and paw temperatures had become stable. Capsaicin (30 $\mu g$ in 3 $\mu l$ over 3 min) was infused into the Nc. caudatus/putamen. The injection cannula was left *in situ* throughout the experiment.

### *Substances*

The stock solution (10 mg $ml^{-1}$) of capsaicin (Merck, Darmstadt, Germany) was made in 10% (v/v) Tween 80 and 10% (v/v) ethanol in saline. Colchicine (Sigma, St. Louis, MO., U.S.A.) was dissolved in saline. Diazepam (Valium) was obtained from Hoffmann-La Roche (Vienna, Austria). (+)-MK-801 (Dizocilpine maleate) from Research Biochemical Inc. (Natick, Mass., U.S.A.), was dissolved in saline. Pentobarbitone sodium (Nembutal) was obtained from Sanofi (Libourne, France). The stock solution of substance P (Peninsula, Heidelberg, Germany) was made in 0.01 M acetic acid and diluted with saline. SR-140333 ((S)1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)-piperidin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2.2.2.]octane, chloride), a gift from Sanofi Recherche (Montpellier, France), was dissolved in dimethylsulphoxide at a concentration of 10 mg $ml^{-1}$ and diluted with saline.

### *Statistical analysis*

The results are expressed as mean values ± s.e.mean. The significance of differences between the different treatment groups was evaluated by the Mann-Whitney *U* test. The significance of changes from baseline values of mean blood pressure and temperature were analyzed by the Quade test (Conover, 1980). Probability values smaller than 0.05 were considered to indicate a significant difference.

## Results

### *Peripheral effects*

Antidromic electrical stimulation of the sciatic nerve led to a pronounced plasma protein extravasation (180 ± 12 mg Evans blue per g wet wt) in the innervated skin area of the hind paws of saline-treated rats (Figure 1a). Pretreatment with colchicine inhibited this plasma protein extravasation by 33%. In rats pretreated with SR-140333 (10, 30 or 100 $\mu g\ kg^{-1}$), the plasma protein leakage was reduced by 36%, 85%, and 86%, respectively, (Figure 1a). MK-801 did not influence plasma protein extravasation (176 ± 13 $\mu g\ g^{-1}$, $n = 6$, data not shown).

The reduction of mustard oil-induced plasma extravasation in colchicine-treated rats was within the range of that observed with antidromic sciatic nerve stimulation (Figure 1b). SR-140333 inhibited the plasma protein extravasation significantly at a dose of 30 $\mu g\ kg^{-1}$ by 85%, at 100 $\mu g\ kg^{-1}$ by 86% (Figure 1b).

The plasma protein extravasation evoked by substance P (13 $\mu g\ ml^{-1}$, i.v.) was comparable in rats treated with colchicine and in their controls (Figure 1c). SR-140333, at doses of 30 and 100 $\mu g\ kg^{-1}$, inhibited the response to substance P by 45% and 74%, respectively, (Figure 1c).

### *Central effects*

There were no significant differences in the basal mean arterial pressure between the different experimental groups of rats (127 ± 4 mmHg). Capsaicin, infused i.a., induced a dose-dependent reflex fall in blood pressure in the vehicle-treated rats (Figure 2). Colchicine pretreatment significantly reduced the depressor response induced by capsaicin. Pretreatment with SR-140333 (100 $\mu g\ kg^{-1}$) did not affect the capsaicin-induced fall in blood pressure (Figure 2). MK-801 has been shown by Donnerer & Amann (1994) to block the depressor reflex response to capsaicin completely.

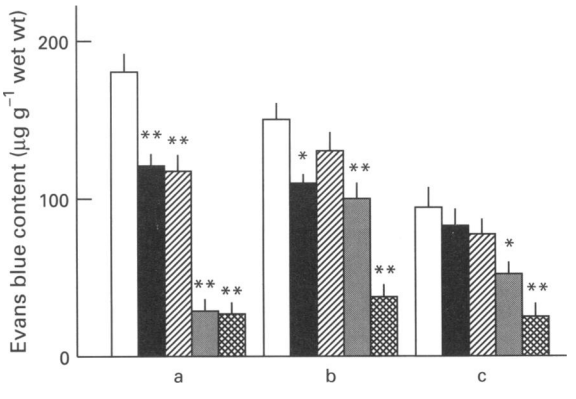


**Figure 1** Plasma protein extravasation in the dorsal skin of the rat hind paw induced by (**a**) antidromic stimulation (5 V, 2 ms, 10 Hz, 5 min) of the distal end of the severed sciatic nerve; (**b**) topical application of mustard oil (5% w/w in liquid paraffin) 3 times at 5 min intervals; and (**c**) injection of substance P (13 $\mu g\ kg^{-1}$, i.v.). All rats were anaesthetized with sodium pentobarbitone (60 mg $kg^{-1}$, i.p.). Influence of drugs on the induced extravasation: Control (open columns), colchicine (120 $\mu g\ kg^{-1}$ $day^{-1}$, i.p., for 3 days; solid columns), SR-140333 (10 $\mu g\ kg^{-1}$: hatched columns; 30 $\mu g\ kg^{-1}$: shaded columns; 100 $\mu g\ kg^{-1}$: cross-hatched columns; all doses given i.v. 5 min before stimulation of plasma protein extravasation). Evans blue (**a**,**b**: 25 mg $kg^{-1}$; **c**: 50 mg $kg^{-1}$) was injected i.v. 10 min before the stimuli. Treatment with MK-801 (100 $\mu g\ kg^{-1}$, i.v.) 10 min before the stimuli had no effect (data not shown). Mean values ± s.e.mean, $n \geq 6$ per group. Significance of difference from controls: $^*P < 0.05$, $^{**}P < 0.01$ (Mann-Whitney U test).

Similar results were obtained when the ascending colon was distended. In the control group, the increase in intraluminal pressure resulted in a reflex fall in the blood pressure (Figure 3). The magnitude of the fall in blood pressure was related to the intraluminal pressure applied. In rats treated with colchicine or MK-801, a significant inhibition of the depressor response was found. SR-140333 (100 $\mu g\ kg^{-1}$) had no effect on this reflex (Figure 3).

Electrical stimulation of afferent fibres in the sciatic nerve caused a voltage-dependent fall in blood pressure in vehicle-treated rats (Figure 4). Colchicine pretreatment did not reduce the depressor reflex response significantly. SR-140333 did not influence the size of the depressor reflex response, whereas MK-801 caused a significant inhibition.

All three depressor reflex responses were abolished 30 min after the s.c. injection of 30 mg $kg^{-1}$ capsaicin (Griesbacher, 1994). In the present study, this test was carried out at the end of most experiments.

The findings described here show that SR-140333, even at the high dose of 100 $\mu g\ kg^{-1}$, did not inhibit any of the depressor reflexes involving capsaicin-sensitive afferent neurones. Colchicine significantly reduced the depressor responses to capsaicin and to colon distension, but did not affect the depressor response to electrical stimulation of the proximal segment of the severed sciatic nerve. MK-801 effectively inhibited all three depressor reflexes.

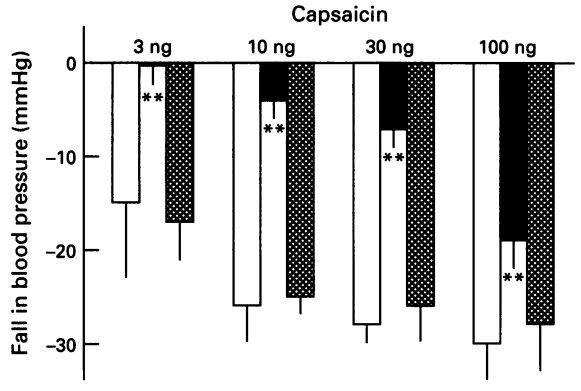


**Figure 2** Depressor reflex induced by successive i.a. infusions of 3, 10, 30 and 100 ng capsaicin (capsaicin solution 1 ng $\mu l^{-1}$; infusion rate 1 $\mu l\ s^{-1}$). All rats were anaesthetized with sodium pentobarbitone (60 mg $kg^{-1}$, i.p.). Control rats (open columns), rats pretreated with colchicine (120 $\mu g\ kg^{-1}\ day^{-1}$, i.p., for 3 days: solid columns), rats pretreated with SR-140333 (100 $\mu g\ kg^{-1}$, i.v., 5 min before the start of the capsaicin infusion: cross-hatched columns). One group of rats was treated with MK-801 (100 $\mu g\ kg^{-1}$, i.v.) 10 min before capsaicin; this blocked the effect of capsaicin completely (results not shown). Mean values ± s.e.mean, $n \geq 6$ per group. Significance of difference from controls: $^{**}P < 0.01$ (Mann-Whitney U test).

## *Capsaicin-induced heat dissipation*

The effect of capsaicin, administered either i.v. or intracerebrally, on paw skin temperature (fore and hind paws) and on colon temperature was investigated. In control animals, the injection of capsaicin (200 $\mu g\ kg^{-1}$, i.v.) resulted in a temperature rise which was larger in the fore paws than in the hind paws; it reached a maximum within 2–4 min and remained increased for 8 min. Colon temperature was decreased within 4–10 min after the injection and remained low for at least 10 min.

In colchicine-pretreated rats, the increase in fore paw skin temperature after i.v. injection of capsaicin was not significantly different from that of control rats (Figure 5). Similarly, the capsaicin-induced decrease in colon temperature of the colchicine-pretreated rats was the same as in vehicle-treated animals (data not shown). Treatment with SR-140333 completely inhibited the rise in fore paw temperature in response to capsaicin, whereas MK-801 did not (Figure 5).

Infusion of capsaicin into the Nc. caudatus/putamen evoked an increase in the temperature of the skin of both the fore and the hind paws and a decrease in colon temperature (Figure 6). Colchicine treatment or the administration of SR-140333 did not influence the capsaicin-evoked rise in paw temperature (data not shown); MK-801 completely prevented it (Figure 6).

## Discussion

### *Effects of colchicine, SR-140333 and MK-801 on functions involving the peripheral terminals of small diameter afferent nerve fibres*

After three days of colchicine treatment, the plasma protein extravasation induced by antidromic sciatic nerve stimulation

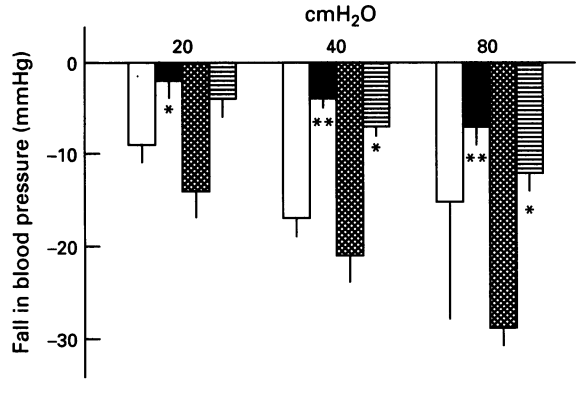


**Figure 3** Depressor reflex induced by successive increases of the intraluminal pressure (+20, +40 and +80 $cmH_2O$) of the ascending colon for 10 s at 5 min intervals. All rats were anaesthetized with sodium pentobarbitone (60 mg $kg^{-1}$, i.p.). Control rats (open columns), rats pretreated with colchicine (120 $\mu g\ kg^{-1}\ day^{-1}$, i.p., for 3 days: solid columns), rats pretreated with SR-140333 (100 $\mu g\ kg^{-1}$, i.v., 5 min before the start of colon distention: cross-hatched columns), rats treated with MK-801 (100 $\mu g\ kg^{-1}$, i.v., 10 min before colon distension: horizontally lined columns). Mean values ± s.e.mean, $n \geq 6$ per group. Significance of difference from controls: $^{*}P < 0.05$, $^{**}P < 0.01$ (Mann-Whitney U test).

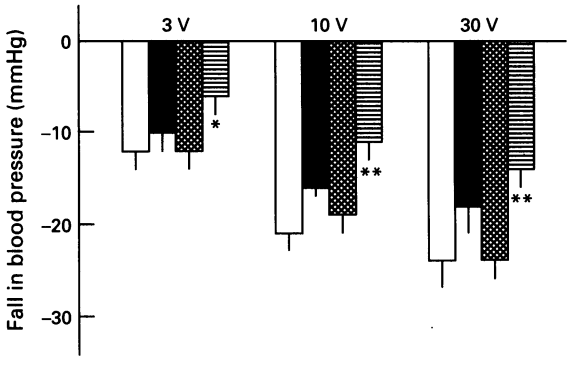


**Figure 4** Depressor reflex induced by successive electrical stimulations (1 ms, 5 Hz; 3, 10 and 30 V) of the proximal end of the severed sciatic nerve. All rats were anaesthetized with pentobarbitone sodium (60 mg $kg^{-1}$, i.p.). Control rats (open columns), rats pretreated with colchicine (120 $\mu g\ kg^{-1}\ day^{-1}$, i.p., for 3 days: solid columns), rats pretreated with SR-140333 (100 $\mu g\ kg^{-1}$, i.v., 5 min before 1st stimulation: cross-hatched columns), rats treated with MK-801 (100 $\mu g\ kg^{-1}$, i.v., 10 min before 1st stimulation: horizontally lined columns). Mean values ± s.e.mean, $n \geq 6$ per group. Significance of difference from controls: $^{*}P < 0.05$, $^{**}P < 0.01$ (Mann-Whitney U test).

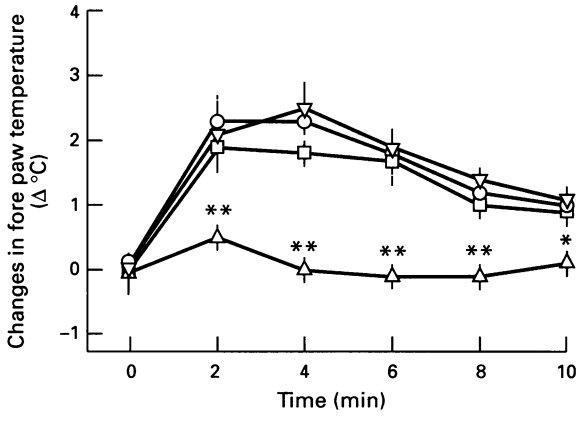


**Figure 5** Effect of capsaicin (200 $\mu g\,kg^{-1}$, i.v.), injected at time zero, on skin temperature of forepaws of control rats (○), rats treated with colchicine (120 $\mu g\,kg^{-1}\,day^{-1}$, i.p., for 3 days: □), rats treated with SR-140333 (100 $mg\,kg^{-1}$, i.v., 5 min before the capsaicin injection: △) and rats treated with MK-801 (100 $mg\,kg^{-1}$, i.v., 10 min before capsaicin: ▽). All rats were sedated with diazepam (25 $mg\,kg^{-1}$, i.p.). Mean values ± s.e.mean, $n \geq 6$ per group. Significance of difference from controls: $^*P<0.05$, $^{**}P<0.01$ (Mann-Whitney U test).

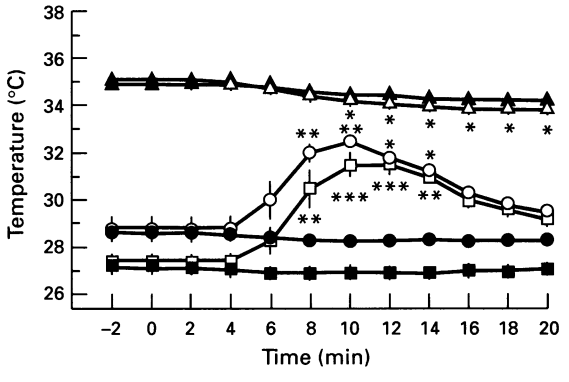


**Figure 6** Skin temperature of fore paws (○,●) and hind paws (□,■), and colon temperature (△,▲) following an intracerebral infusion of capsaicin in control rats (open symbols) and in rats treated with MK-801 (100 $\mu g\,kg^{-1}$, i.v., 10 min before capsaicin: solid symbols). The rats were anaesthetized with chloral hydrate (400 $mg\,kg^{-1}$, i.p.). Capsaicin (30 $\mu g$ in 3 $\mu l$ over 3 min) was infused into the Nc. caudatus/putamen starting at time zero. Treatment with colchicine (120 $\mu g\,kg^{-1}\,day^{-1}$, i.p., for 3 days) or with SR-140333 (100 $\mu g\,kg^{-1}$, i.v., 5 min before the experiment) did not alter the temperature-effects of the i.c. infusion of capsaicin (results not shown). Mean values ± s.e.mean, $n \geq 6$ per group. Significance of difference from basal values: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (Quade test).

or by topical application of mustard oil was diminished; the latter somewhat less than the former. In our initial experiments, in which we used Sprague-Dawley rats, the inhibitory effect of colchicine was smaller than that found in Wistar rats by Moussaoui *et al.* (1993). Therefore, we repeated the experiments with Wistar rats, but no difference between the two strains in the effect of colchicine was found. Plasma protein extravasation induced by the two methods was dose-dependently reduced by SR-140333. In contrast, MK-801 had no effect on plasma protein extravasation evoked by antidromic sciatic nerve stimulation. The effect of substance P i.v. was, as expected, inhibited by SR-140333 but not by colchicine. These results confirm the work of Moussaoui *et al.* (1993) with RP 67580, of Emonds-Alt *et al.* (1993) with SR-140333, and Lembeck *et al.* (1992) with CP-96,345. It can, therefore, be concluded that substance P, together with neurokinin A and CGRP, are the essential mediators at the peripheral terminals of afferent C-fibres.

### *Effects of colchicine, SR-140333 and MK-801 on depressor reflexes*

The depressor reflexes used have been shown to be mediated by capsaicin-sensitive afferent neurones (for review see Holzer, 1991). Griesbacher (1994) observed desensitization to capsaicin as soon as 30 min after the s.c. injection of 30 mg $kg^{-1}$ capsaicin. By using this procedure, we obtained a complete inhibition of all three reflexes. The involvement of capsaicin-sensitive afferent neurones in these reflexes has, therefore, been confirmed.

After treatment with colchicine, the depressor reflexes induced by capsaicin i.a. or by colon distension were greatly reduced, but not the depressor reflex response evoked by afferent sciatic nerve stimulation. The afferent stimulation of the sciatic nerve also involves nerve fibres other than the small diameter fibres. That there was only a minor inhibition of the axonal flow of neurotransmitters in these other fibres could be a possible explanation for the lack of an effect of colchicine on this reflex.

None of the depressor reflexes was inhibited by SR-140333 (even at the high dose of 100 $\mu g\,kg^{-1}$). SR-140333, given i.p. to mice inhibited the scratching response induced by i.c.v. injected substance P or apomorphine and also abolished the facilitation of the tail-flick response to noxious heat in rats (Jung *et al.*, 1994). Its penetration into the CNS can therefore be assumed. Its inability to inhibit the investigated reflexes will be discussed later.

MK-801, at a dose 25 fold higher (2.5 mg $kg^{-1}$, i.v.) than that used in our experiments, induced no change in blood pressure of pentobarbitone-anaesthetized rats (Monteau *et al.*, 1990). MK-801 (100 mg $kg^{-1}$, i.v.) inhibited all three depressor reflexes. Complete inhibition of the reflex response induced by capsaicin i.a. has already been shown by Donnerer & Amann (1994). The depressor reflex responses to colon distension or to afferent sciatic nerve stimulation were significantly reduced, but not completely abolished, by MK-801. Inhibition of these reflexes indicates that MK-801 affects central transmission in the reflex arcs. It did not, however, inhibit the effect of antidromic sciatic nerve stimulation. The effect of capsaicin (200 $\mu g$, i.v.) was completely blocked by SR-140333, but not influenced by MK-801. This excludes an effect of MK-801 at peripheral nerve terminals (see above).

### *Temperature regulation*

Stimulation of the afferent fibres by capsaicin i.v. (200 $\mu g$) induced an increase in paw skin temperature of about 2°C; the effect was more pronounced in fore than in hind paws, an observation not previously recorded. Treatment with colchicine or MK-801 did not affect the rise in fore paw skin temperature, whereas SR-140333 (100 $\mu g\,kg^{-1}$) completely prevented the increase in temperature of the fore paws. This effect of SR-140333 can be explained by the inhibition of vasodilatation resulting from the capsaicin-induced release of substance P from peripheral terminals of afferent nerve fibres (Figure 5). It should be mentioned that capsaicin i.v. not only induces cutaneous vasodilatation, but also activates a reflex which diminishes cutaneous adrenergic vasoconstrictor tone (Donnerer & Lembeck, 1983).

An infusion of capsaicin (30 $\mu g$ over 3 min) into the Nc. caudatus/putamen resulted in a rise in temperature of the paws for several minutes (Figure 6). The response to intracerebrally injected capsaicin was completely inhibited following treatment with a large dose of capsaicin (0.5–20 mg $kg^{-1}$, s.c.; Hajós *et al.*, 1986). The response to intracerebrally injected capsaicin was not influenced by colchicine pretreatment which contrasts with its effect on the depressor reflex. SR-140333 was without an effect on the increase in skin temperature induced

by intracerebral capsaicin. This can be explained if this receptor blocker acts only in the periphery. MK-801 totally prevented the rise in skin temperature in response to intracerebrally administered capsaicin. A direct effect of MK-801 (100 $\mu g\ kg^{-1}$, i.v., 10 min before capsaicin) on temperature regulation can be excluded as the baseline temperatures of paws and colon were the same as in the control groups (Figure 6).

### *Co-operation between neurokinins and excitatory amino acid neurotransmitters*

In 1977, Otsuka & Konishi using the isolated spinal cord preparation from new born rats, observed that neurones adjacent to the central terminals of afferent fibres could be stimulated by glutamate as well as by substance P. The release of substance P, neurokinin A and other peptides from central nerve terminals in the spinal cord following the stimulation of small diameter afferent nerve fibres has also been demonstrated with several other methods (for reviews see Pernow, 1983; Otsuka & Yoshioka, 1993). The extinction of various autonomic reflexes and neuroendocrine regulation mechanisms following capsaicin pretreatment were therefore attributed to the depletion of peptide neurotransmitters from afferent fibres (for review see Lembeck, 1988).

An earlier attempt to show the release of glutamate from the central terminals of small diameter afferent nerve fibres in spinal cord slices resulted in the demonstration of a release of glutamate by depolarization with $K^+$ or veratridine. No release of glutamate was induced by capsaicin. The released glutamate was, therefore, thought to originate from neurones that were capsaicin-insensitive (Donnerer, 1991). However, recent experiments with a new, highly sensitive method for the estimation of glutamate (Ueda *et al.*, 1994) have provided evidence for a capsaicin-induced release of glutamate in parallel with the release of substance P from afferent C-fibre terminals in the spinal cord.

These findings illuminate the functional significance of many earlier results. The co-existence of substance P and excitatory amino acids in primary afferent fibre terminals in the spinal cord (Battaglia & Rustioni, 1988; De Biasi & Rustioni, 1988; Merighi *et al.*, 1991), the involvement of receptors for substance P and NMDA in the transmission of nociceptive signals (Dickenson & Sullivan, 1990; Woolf & Thompson, 1991), the modulation of glutamate-induced current flow in spinal dorsal horn neurones by substance P (Randic *et al.*, 1990), and the participation of receptors for substance P and NMDA in the central sensitization of the nociceptive pressor reflex (Xu *et al.*, 1992a).

Responses to NMDA and substance P have been found to be cross-sensitive to their respective antagonists, as demonstrated in the rat spinal cord by Brugger *et al.* (1990). Thus, ventral root responses in the rat isolated spinal cord preparation evoked by stimulation of afferent nerve fibres were hardly affected by antagonists of NMDA or tachykinin receptors alone, but there was a pronounced inhibition when both types of antagonists were used together (Thompson *et al.*, 1993). Activation of $NK_1$ or $NK_2$ receptors enhanced NMDA- and quisqualate-evoked ventral root depolarization (Urban *et al.*, 1994a). The co-operation between neurokinin and excitatory amino acid transmitters has recently been reviewed by Urban *et al.* (1994b).

There is a discrepancy between the effects of neurokinin receptor antagonists on functions involving the peripheral terminals and those involving the central terminals of primary afferent fibres in experiments *in vivo*. All effects of the release of neurotransmitters from peripheral terminals were completely inhibited by neurokinin $NK_1$ receptor antagonists (CP-96,345: Lembeck *et al.*, 1992; SR-140333: Emonds-Alt *et al.*, 1993; RP-67580: Moussaoui *et al.*, 1993). No information is available about the release of excitatory amino acids from the peripheral terminals of C-fibres. Inhibition of functions involving the central terminals of afferent nerve fibres following the systemic administration of tachykinin antagonists could explain some of the following observations. The depressor reflex to i.a. capsaicin was inhibited by CP-96,345; however, an inhibition of the same extent was observed following the administration of the inactive enantiomer, CP-96,344. This effect is, therefore, unspecific (Griesbacher *et al.*, 1992). The second phase of the response in the formalin test was inhibited by CP-96,345 (200 $\mu g$, intrathecally), but not by its inactive enantiomer. Motor dysfunction was induced by both enantiomers at a dose of 400 $\mu g$, intrathecally. CP-96,345 (5 and 15 $mg\ kg^{-1}$, i.p.) depressed the second phase of the response in the formalin test, but CP-96,344 was not tested (Yamamoto & Yaksh, 1991). CP-96,345 (up to 2.4 nmol, intrathecally) antagonized the substance P-evoked, C-fibre-mediated, facilitation of the flexor reflex (Xu *et al.*, 1992b), but neither CP-96,344 nor the effect of systemic administration was tested. Intrathecal administration of RP-67580 (1 – 10 $\mu g$) resulted in inhibition of the response in the formalin test, whereas the administration of its inactive enantiomer, RP-68651, did not; the effects of systemic administration were not investigated (Chapman & Dickenson, 1993). The reflex responses to distension of the rectum were reduced by CP-96,345 (5 – 10 $mg\ kg^{-1}$, i.p.) and by RP-67580 (0.2 $mg\ kg^{-1}$, i.p.), but the effects of the inactive enantiomers were not studied (Julia *et al.*, 1994). The conditioning stimulus facilitation of a spinal flexion reflex response to nociceptor stimulation was attenuated by RP-67580 (2.5 $mg\ kg^{-1}$, i.v.), but not by its inactive enantiomer (up to 3 $mg\ kg^{-1}$, i.v.; Laird *et al.*, 1993). The analgesic action of RP-67580, administered i.p., has been shown in two tests (Garret *et al.*, 1991).

Evidence for the penetration of SR-140333 into the CNS is provided by the investigation of Jung *et al.* (1994) mentioned above. This compound seemed, therefore, suitable for the present study. However, we found no evidence for a specific central action following its systemic administration, even at a dose that had a full effect in the periphery. It seems, therefore, that central transmission mediated by substance P could be involved in functions other than those investigated here, where the inhibition by MK-801, an antagonist at NMDA receptors, was evident.

The simultaneous release of glutamate and substance P from central terminals of afferent fibres, as demonstrated by Ueda *et al.* (1994), and as indicated by our present findings, requires a reconsideration of the role of substance P in the central transmission of signals conveyed by afferent fibres. Excitatory amino acids could play a dominant role and peptides might serve only as modulators. In addition, reflexes mediated by capsaicin-sensitive afferent nerve fibres are exposed to many other influences: They are depressed by galanin, somatostatin and GABA, and potentiated by CGRP (Nussbaumer *et al.*, 1989). The reflex response to i.a. capsaicin is decreased following chronic administration of morphine and enhanced after sudden morphine replacement by naloxone (Donnerer, 1989). The reflex response to i.a. capsaicin is also inhibited by clonidine acting at a supraspinal level (Donnerer *et al.*, 1988). All of these factors could affect the transmission by glutamate or by substance P at the first central synapse.

**Note added in proof**

A decrease of the capsaicin-induced release of glutamate from afferent nerve terminals in the rat spinal cord by clonidine (Ueda, M., Oyama, T., Kuraishi, Y., Akaike, A. & Satoh, M. (1995). *Neurosci. Lett.*, **188,** 137 – 139) and by morphine (Ueda, M., Sugimoto, K., Oyama, T., Kuraishi, Y. & Satoh, M., (1995). *Neuropharmacol.*, **34,** 303 – 308) has been found recently. These findings confirm and explain earlier results of Donnerer *et al.* (1988, 1989) on the depressor reflex.

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