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Release of immunoreactive substance P in the brain stem upon stimulation of the cranial dura mater with low pH – inhibition by the serotonin (5-HT_1) receptor agonist CP 93,129

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1 The therapeutical benefit of serotonin (5-HT_1) receptor agonists in the treatment of migraine headache has been attributed to their inhibitory effect on the release of pro-inflammatory neuropeptides from trigeminal afferents within the cranial meninges. The effect of 5-HT_1 receptor agonists on the release of neuropeptides from central afferent terminals has not been examined so far. In the present study in the rat we therefore measured the effect of the 5-HT_{1B} receptor agonist CP 93,129 on the stimulation-evoked release of immunoreactive substance P (ir-SP) in the spinal trigeminal nucleus.

2 To measure release of ir-SP, microprobes coated with antibody to substance P were inserted into the medulla oblongata at the level of the obex. The ipsilateral parietal dura mater encephali was exposed and stimulated with acid phosphate buffered Tyrode solution (pH 5.8). This chemical stimulus increased the release of ir-SP in the medullary dorsal horn.

3 Systemic (i.v.) administration of CP 93,129 (460 nmol kg^{-1}) prior to stimulation suppressed the stimulation-evoked increase of release of ir-SP. Local administration of CP 93,129 (10 μM) to the dorsal surface of the medulla had no significant inhibitory effect on the release.

4 It is concluded that systemically applied 5-HT_1 receptor agonists reduce the stimulation-evoked release of substance P from the central endings of meningeal afferents in the spinal trigeminal nucleus (medullary dorsal horn). This inhibitory effect may contribute to the antinociceptive effect of 5-HT_1 receptor agonists in migraine.

Keywords: 5-Hydroxytryptamine receptor agonist; neuropeptides; substance P; dura mater encephali; meningeal afferents; medullary dorsal horn; trigeminal nucleus; migraine pain

Introduction

The dura mater encephali is richly innervated by thin afferent nerve fibres, a minor proportion of which exhibits substance P immunoreactivity (rat: Keller & Marfurt, 1991 and Von Düring *et al*., 1990; cat and rat: Messlinger *et al*., 1993; guinea-pig: Edvinsson *et al*., 1983). Meningeal afferents originating in the trigeminal ganglion (Mayberg *et al*., 1984; O'Connor & van der Kooy, 1986; Steiger & Meakin, 1984; Uddman *et al*., 1989) project mainly to the spinal trigeminal nucleus, where numerous substance P immunoreactive nerve fibres were found particularly in the substantia gelatinosa (Cuello *et al*., 1978; Del Fiacco *et al*., 1983; Pearson & Jennes, 1988; Tashiro *et al*., 1991).

Substance P has been implicated in the nociceptive processing within the spinal cord and the brain stem. Substance P receptor-immunoreactive neurons were identified in the rat spinal cord and spinal trigeminal nucleus (Brown *et al*., 1995). In spinal cord neurons the activation of neurokinin-1 receptors by substance P may be involved in the transmission of nociceptive information (De Koninck *et al*., 1992) and in the inflammation/injury-evoked development of hyperexcitability (Neugebauer *et al*., 1995; Thompson *et al*., 1994). Henry *et al*. (1980) have shown that neurons in the spinal trigeminal nucleus of the cat are excited by ionophoretic administration of substance P.

Electrophysiological recordings in the rat and the cat demonstrated that meningeal afferents and neurons in the spinal trigeminal nucleus with meningeal afferent input can be activated upon electrical, mechanical and chemical stimulation of the dura mater (Bove & Moskowitz, 1997; Davis & Dostrovsky, 1986, 1988; Ebersberger *et al*., 1997; Strassman *et al*., 1986, 1996). An increase in c-*fos* expression was observed in the spinal trigeminal nucleus following noxious stimulation of the cranial meninges (Cutrer *et al*., 1995; Kaube *et al*., 1993; Nozaki *et al*., 1992). Substance P, which may be released in the trigeminal nucleus upon meningeal stimulation, possibly contributes to these nociceptive responses. We have recently found pronounced release of immunoreactive substance P (ir-SP) in the medullary brain stem after the application of acidic phosphate buffer to the exposed dura mater in the rat (Schaible *et al*., 1997). This acidic stimulus is able to activate a major proportion of neurons in the spinal trigeminal nucleus (Messlinger *et al*., 1995).

From classical intraoperative experiments (Ray & Wolff, 1940; Penfield & McNaughton, 1940) there is strong evidence that the sensation of headache caused by afferent stimulation depends on the activation of nociceptors located in the dura mater encephali, although the stimuli which activate meningeal nociceptors in headache syndromes are not known. In the treatment of migraine pain and cluster headache, serotonin (5-HT_1) receptor agonists are used with considerable success (Ferrari, 1993; Goadsby & Edvinsson, 1994; Wilkinson *et al*., 1995). The precise mechanism and location of the antinociceptive action of 5-HT_1 receptor agonists is unclear. There is evidence, however, that they affect the release of neuropeptides

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such as substance P and calcitonin gene-related peptide (CGRP) from peripheral endings of trigeminal afferents. 5-HT_1 receptor agonists were shown to inhibit the increase of CGRP concentration in the venous outflow from the head both after experimental trigeminal ganglion stimulation and during migraine attacks (Buzzi *et al*., 1991; Goadsby & Edvinsson, 1993, 1994) and to suppress the expression of c-*fos* in the trigeminal brain stem after noxious stimulation of trigeminal structures (Cutrer *et al*., 1995; Nozaki *et al*., 1992; Shepheard *et al*., 1995).

In the present study we therefore examined whether CP 93,129, a potent and selective agonist of the 5-HT_{1B} receptor in the rat (Macor *et al*., 1990), is able to inhibit the release of ir-SP in the medullary trigeminal brain stem following chemonociceptive stimulation of the dura mater. CP 93,129 has previously been shown to be highly effective in reducing the substance P-dependent plasma extravasation in the rat dura mater (Matsubara *et al*., 1991) and the c-*fos* expression in the spinal trigeminal nucleus (Nozaki *et al*., 1992).

Methods

Preparation of antibody microprobes

Antibody microprobes were prepared according to Duggan *et al*. (1988). Briefly, borosilicate micropipettes were heat sealed at both ends and then incubated in a 10% solution of aminopropyltriethoxysilane in toluene. Subsequent heat curing produced a siloxane polymer on the outer surface. Using glutaraldehyde, protein A was immobilized to the siloxane polymer on the surface of the microprobes. Then the tips of the microprobes were introduced overnight into glass capillaries containing an antiserum to substance P (SP; Peninsula; specificity 100% for SP, SP3-11, SP4-11, SP5-11; cross-reactivity for other neurokinins $\leqslant 0.01\%$). The sensitivity of the antibody coated microprobes was tested *in vitro*. The ends of the microprobes were kept overnight at 6°C in 5 μl capillaries containing a PBS-azide solution of ^{125}I-radiolabelled SP (substance P, ^{125}I-labelled with Bolton and Hunter reagent; about 8000 c.p.m. 5 μl^{-1}) and bovine serum albumin. Then they were washed for 15 min in cold phosphate buffered saline (PBS) containing Tween 80 (0.1%). The probes bound about 10–20% of the total radioactivity in the capillary. Preincubation of the tips of the microprobes in SP solutions for 30 min at 37°C prior to incubation in [^{125}I]-SP suppressed the binding of labelled SP to about 20% with SP 10^{-6} M and to about 50% with SP 10^{-7} M.

Anaesthesia and surgery

Twenty-five male Wistar rats (280–420 g, Charles River Wiga, Sulzfeld, Germany) were used. Anaesthesia was induced by an i.p. injection of 120 mg kg^{-1} thiopentone (Trapanal, Byk Gulden, Konstanz, Germany) followed by supplemental doses of 25–30 mg kg^{-1} thiopentone i.p. when required. The animals were tracheotomized but were breathing spontaneously. A gentle jet of oxygen was blown towards the opening of the tracheal cannula to facilitate oxygenation. Catheters were introduced in the femoral artery and vein to measure arterial blood pressure and to inject compounds. Blood pressure and body temperature (regulated by a feedback controlled heating plate) were kept within physiological ranges. Depth of anaesthesia was routinely assessed and held at a level in which the corneal blink reflex was absent and noxious stimuli failed to elicit nociceptive motor reflexes or changes of the systemic arterial pressure (mean 90–110 mm Hg^{-1}). The experiments were terminated by an i.v. overdose of thiopentone.

The head of the animal was fixed in a stereotaxic frame and the scalp was incised. The skull was trepanized using an electric drill while cold Tyrode solution (4°C) was applied to avoid thermal lesions. The parietal bone at one side was slowly abraded while the innermost layer of the bone was removed using fine forceps. The size of this opening was about 8×7 mm. The exposed dura mater encephali was covered with gauze soaked with Tyrode solution (pH 7.4). The skin and the muscles in the neck region were divided along the medial line. The atlanto-occipital ligament and the underlying spinal dura mater were incised, dissected from the occipital bone and pulled aside. To expose the medulla oblongata at the obex region, about 0.5 mm of the occipital bone was removed. With an upright position of the head, the dorsal surface of the exposed brain stem had an angle of 25–30° to the horizontal plane allowing continuous superfusion of the surface by cerebral liquor. The spinal pia mater was kept intact. For orientation and positioning of the microprobes, the shape of the medulla oblongata and the network of pial blood vessels on the exposed area were drawn and x-y-coordinates were added to the sketch with the obex as a reference.

Experimental protocol

Using a microstepper tilted at an angle of 25–30°, microprobes were introduced rectangularly to the dorsal surface into the ipsilateral medulla oblongata. The probes were inserted to a depth of 2.5 mm within a distance of 1.7–3.2 mm lateral from the midline and from 0.5 mm anterior to 0.7 mm posterior to the obex. Within this area the insertion sites were slightly and randomly varied so that the probes were not inserted twice at exactly the same site. All probes were kept in the medulla for 10 min. Before inserting and before removing a probe, the liquor floating on the medulla was sucked off.

In 13 experiments the effect of intravenous CP 93,129 on stimulus-evoked release of ir-SP was assessed. CP 93,129 was freshly dissolved in saline with 5% dimethyl sulfoxide (DMSO) at a concentration of 10 mM and diluted with saline to 460 nmol ml^{-1} for i.v. administration. In the experiments a dose of 460 nmol kg^{-1} was used. The vehicle (DMSO in saline) was diluted in the same way. To obtain a pre-stimulation baseline, in all experiments the first six probes (1–6) were introduced successively before any treatment. Then either CP 93,129 at a dose of 460 nmol kg^{-1} or the corresponding vehicle was injected intravenously. Ten minutes after this injection, the next probes (7–9) were inserted. One minute after inserting each of these three probes, a piece of cotton (8×7 mm in size) was soaked with phosphate buffered acidic Tyrode solution (pH 5.8) and laid on the exposed dura mater. After this stimulation period the acid buffer was replaced by Tyrode solution (pH 7.4) twice, and ten more probes (11–20) were successively introduced without stimulation of the dura. The whole procedure was repeated using a second application of acidic solution to the dura and a second intravenous injection of the vehicle or CP 93,129, respectively.

In 12 experiments, we measured the effect of CP 93,129 applied to the dorsal surface of the brain stem. The protocol of insertion of microprobes and stimulation of the dura was the same as described above. For topical application of the compounds, a piece of cotton soaked with either CP 93,129 at a concentration of 10 μM or with the vehicle was laid on the surface of the medulla oblongata, and 25 μl of the solution were administered twice within a period of 10 min to the

cotton pad. After removal of the cotton from the medulla the probes number 7–9 were introduced (stimulation period). Stimulation of the exposed dura mater was done as in the first series of experiments, but only one stimulation cycle was performed.

Processing of microprobes and analysis of data

After removal from the medulla oblongata, the probes were washed for 15 min in cold PBS containing Tween 80 (0.1%). Then they were incubated overnight at 4°C in 5 μl capillaries containing ^{125}I-radiolabelled SP (^{125}I-SP, labelled with Bolton and Hunter reagent, about 8000 c.p.m. 5 μl^{-1}) and bovine serum albumin in PBS-azide. After incubation the probes were washed for 15 min in PBS-Tween. The shafts of the probes were broken off and the tips were placed in a X-ray cassette on a monoemulsion film (MRB 11, Cea AB, Sweden) for 14 days. The radiographic images of the probes were analysed using a CCD camera and an image analysis system (Imaging Technology PC Vision Plus frame grabber board) run on a PC (Hendry *et al*., 1988). This procedure produced an image of each probe showing the grey density along the tip of the probe. The resulting X-ray images were scanned along the probe in 10 μm intervals and five successive integrals were averaged for calculation of the densities along the probe. Local inhibition of binding of [^{125}I]-SP to the probe by previous binding of SP to the probe in the brain stem produces a zone of reduced grey density at this particular site. These sites of deficits were then related to sites within the brain stem.

For analysis of the data, the probes were first normalized. The grey values of the pixels 420–470, corresponding to an area of the probe that was located just outside the spinal cord, were set to 6000 on the grey scale and all remaining pixels of the probes were then related to this reference area. Then probes were pooled and the means $\pm$ s.e.mean were displayed in graphs which showed the averaged grey densities along the probes in relationship to the depth within the brain stem. In each experimental group we pooled the probes inserted before application of the stimulus ('no stimulation'-probes), the probes inserted during the stimulation ('pH 5.8'-probes) and the probes inserted in the periods 5–45 min, 50–90 min and 95–150 min after chemical stimulation of the dura. Comparisons were made between different groups of probes using the Student's *t*-test at intervals of 50 μm along the probe.

Materials

The SP antiserum was purchased from Peninsula, the radioactive tracer [^{125}I]-SP (substance P, ^{125}I-labelled with Bolton and Hunter reagent) from Amersham, and substance P from Calbiochem. The 5-HT$_{1B}$ receptor agonist CP 93,129 (3-(1,2,5,6-tetrahydropyrid-4-yl)pyrrolo[3,2-*b*]pyrid-5-one) was a gift from Pfizer Inc, Groton, CT, U.S.A.

Results

Systemical administration of vehicle and CP 93,129

The stimulus-evoked release of immunoreactive substance P (ir-SP) in the brain stem was assessed in six experiments after i.v. administration of the vehicle and in seven experiments after i.v. administration of CP 93,129. Figure 1A–D show the release of ir-SP in the brain stem in the experiments in which the vehicle was injected. Figure 1A displays the averaged densitometric image of probes that were in the brain stem before stimulation (no stimulation), and of probes that were inserted into the brain stem during the application of acidic solution onto the dura (pH 5.8). Eleven minutes before the application of the acidic stimulus, the vehicle was injected intravenously. The curves in Figure 1A were not significantly different indicating that on average ir-SP was not released above baseline during the application of acidic solution. However, enhanced release of ir-SP above the baseline level was observed in the period 5–45 min after application of acidic solution to the dura (Figure 1B). Probes inserted during this period showed less grey density than probes inserted before stimulation indicating stimulus-evoked release of ir-SP in the brain stem. The difference was significant in a zone between 0.7 and 1.3 mm below the dorsal surface of the brain stem. This difference is also shown in Figure 2 where the ir-SP released is correlated to the anatomical site in the trigeminal brain stem. Figure 1C,D compare the densitometric images of the 'no stimulation'-probes and of the probes inserted 50–90 min and 95–150 min, respectively, after stimulation of the dura. These data show that the stimulus-evoked release of ir-SP was reversible.

When this protocol was repeated in five rats using a second injection of the vehicle and a second administration of acidic solution to the dura, the result was similar. The level of ir-SP was increased in the period 5–45 min after the administration of acidic solution and showed considerable recovery in the periods 50–90 and 95–150 min after stimulation (data not shown).

The same procedure was used in the experiments in which CP 93,129 was injected intravenously before the acidic solution was administered to the dura (Figure 1E–H). On average, there was no enhanced release of ir-SP over the baseline level during the stimulation period (Figure 1E). After acidic stimulation, release of ir-SP tended to be enhanced near the dorsal surface of the brain stem (Figures 1F–H). The curves in Figure 1G are significantly different just around the surface. However, in the CP 93,129-treated group there was no enhancement of release of ir-SP in that area of the brain stem that showed a pronounced enhancement of release of ir-SP in the vehicle-treated animals (compare Figure 1B and F). Thus CP 93,129 was able to reduce stimulus-evoked release of ir-SP.

While CP 93,129 reduced the release of ir-SP after the first stimulation of the dura, the release of ir-SP was not blocked when a second injection of CP 93,129 was given in six rats before the second stimulation of the dura. The increase of the level of ir-SP was most pronounced in the period 50–90 min after stimulation. The change in the pattern of the densitometric curve was similar to that seen in the vehicle-treated animals (data not shown). The systemic arterial pressure was not significantly changed after the first and the second injection of CP 93,129.

Local administration of vehicle and CP 93,129 to the brain stem

In further experiments we tested whether the application of CP 93,129 onto the dorsal surface of the brain stem alters stimulus-evoked release. In six control experiments the dura was stimulated after application of the vehicle onto the brain stem. In general, the application of acidic solution to the dura caused release of ir-SP which was similar to that observed in the experiments described above. However, the time course of release was different since the maximum of release was seen in the period 50–90 min after application of the acidic solution. The maximum release is shown in Figure 3A. It displays the averaged densitometric image of probes inserted before

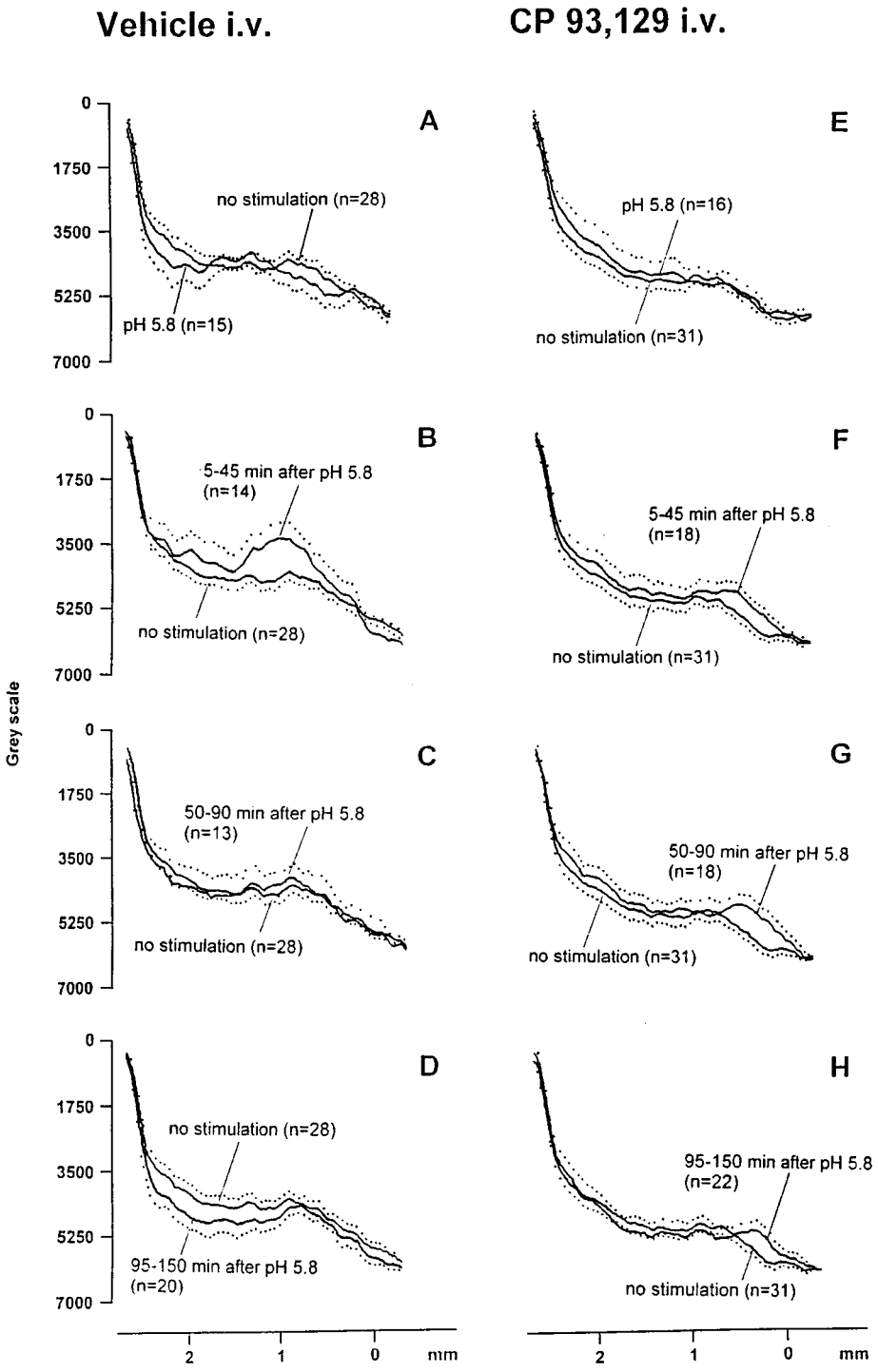

Figure 1 Release of ir-SP in the spinal trigeminal brain stem after the administration of acidic solution to the exposed dura mater. (A–D) Release of ir-SP in animals that received an intravenous injection of the vehicle before the acidic solution was applied. (E–H) Release of ir-SP in animals that received an intravenous injection of 460 nmol kg^{-1} CP 93,129 before the acidic solution was administered. Each curve shows the average densitometric image of microprobes inserted into the medulla oblongata under the conditions indicated. A and E compare the probes inserted before administration of acidic solution (no stimulation) and the probes inserted during the stimulation period. B and F display the mean densities on the grey scale of the 'no stimulation'-probes and the probes inserted 5–45 min after acidic stimulation. In B the difference between these two curves is significant in a depth of 0.7–1.3 mm ($P<0.05$, Student t-test). C and G, and D and H, respectively, compare the 'no stimulation'-probes and the probes inserted 50–90 and 95–150 min after the administration of acidic solution. In A–H, each continuous line represents the mean densitometric analysis of a group of microprobes which have bound [^{125}I]-SP and produced autoradiographic images. The densitometric analysis was performed at 50 μm intervals. The dots represent the standard errors of the mean at each analysis point. Prior to incubation in [^{125}I]-SP the probes were inserted into the medulla oblongata to a depth of 2.5 mm from the dorsal surface for periods of 10 min. Abscissa: depth in the medulla, 0 corresponds to the surface. Ordinate: grey scale integrals obtained by transverse analysis of microprobe images in 50 μm steps. The general increase of the grey density from the tip (at 2.5 mm) towards 1 mm results from the increasing number of bindings sites along the shaft progressing away from the tip. Local inhibition of binding of [^{125}I]-SP (reduced grey density) on the shaft is equated with previous binding of (unlabelled) endogenous ir-SP in the medullary brain stem.

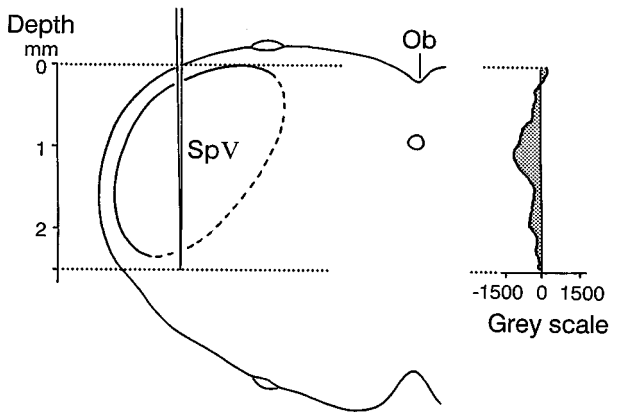

Figure 2 Left panel: Cross section through the left medulla oblongata parallel to the direction of microprobe insertions at the level of obex (Ob). A microprobe is shown in a typical position penetrating the spinal trigeminal nucleus (SpV) to a depth of 2.5 mm. Right panel: Difference profile of the distribution of ir-SP (indicated as reduction in grey density) along a virtual microprobe representing the difference of the mean grey values of a group of 'no stimulation' probes and probes inserted 5–45 min after stimulation with pH 5.8 (same groups as shown in Figure 1B). The difference is maximal at a depth of about 1 mm.

stimulation and of probes inserted 50–90 min after the stimulus. Reduced grey density was mainly found at a depth between 1 and 2 mm. The stimulus-evoked release of ir-SP was not prevented in the experiments in which CP 93,129 was administered to the brain stem ($n=6$). Figure 3B shows the maximum release of ir-SP, which was observed in the period 95–150 min after stimulation with acidic solution.

Discussion

In agreement with our previous study (Schaible *et al*., 1997), the administration of acidic solution to the exposed dura mater evoked enhanced release of ir-SP in the medullary brain stem. In experiments in which the 5-HT_{1B} receptor agonist CP 93,129 was injected intravenously before the application of the stimulus, there was no significant release of ir-SP in the brain stem.

The basic observations on the release of ir-SP in the vehicle-treated rats are consistent with our previous findings (Schaible *et al*., 1997). Firstly, the application of acidic solution to the dura was a potent stimulus to cause release of ir-SP. This is in agreement with the finding that acidic solutions activate and sensitize a high proportion of primary meningeal afferents (Bove & Moskowitz, 1997; Strassman *et al*., 1996) and a major proportion of neurons in the spinal trigeminal nucleus with input from the dura mater in the rat (Messlinger *et al*., 1995). Secondly, enhanced release of ir-SP was mainly observed after and not during the stimulation period indicating a prolonged period of release (see Discussion in Schaible *et al*., 1997). Thirdly, enhanced release of ir-SP was most pronounced in deeper layers of the spinal trigeminal nucleus.

We have used a dose of CP 93,129 that has produced significant effects in previous studies. CP 93,129 at this dose significantly reduced the neurogenic plasma extravasation in the dura mater encephali of the rat (Matsubara *et al*., 1991) which is known to depend on the activation of NK-1 receptors by substance P and (possibly) neurokinin A (Markowitz *et al*., 1987; Buzzi & Moskowitz, 1990). CP 93,129 at this dose also reduced the c-*fos* expression in the spinal trigeminal nucleus of

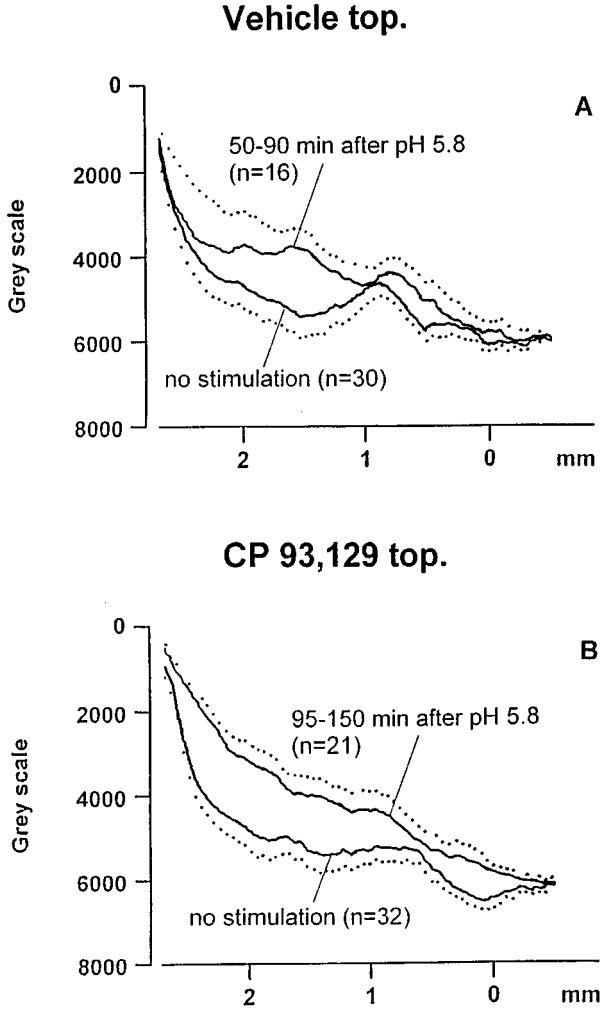

Figure 3 Release of ir-SP in the spinal trigeminal brain stem evoked by administration of acidic solution to the dura mater. (A) Maximum release of ir-SP in rats in which the vehicle was administered topically to the dorsal surface of the medulla oblongata. (B) Maximum release of ir-SP in rats in which CP 93,129 (10 μM) was administered to the medulla. Display as in Figure 1.

the rat evoked by injection of autologous blood into the cisterna magna as a noxious stimulus (Nozaki *et al*., 1992). In the present study there was no enhancement of the release of ir-SP in the medullary brain stem following acidic stimulation after pretreatment with CP 93,129 at this dose. Interestingly, after the second injection of CP 93,129 significant stimulus-evoked release of ir-SP was measured. The reason for this lack of effect is not yet clear. One possibility is that the drug shows tachyphylaxis upon repeated application. It is also possible that processes of sensitization developed in the preparation that caused release of SP by mechanisms which cannot be influenced by $5HT_{1B}$ receptors. To our knowledge effects of repeated application(s) of CP 93,129 have not been reported in other studies.

At present the precise mode and site of action of CP 93,129 and other 5-HT_1 receptor agonists are not fully understood. Probably these compounds bind both to the peripheral and central processes of meningeal afferents. Indeed, trigeminal ganglion cells of different species including man show gene expression of 5-HT_1 receptors (Adham *et al*., 1997; Bruinvels *et al*., 1992; Rebeck *et al*., 1994), and binding of isotopes of 5-HT_1 receptor agonists ([^{3}H]-sumatriptan and [^{3}H]-zolmitriptan) was autoradiographically detected in the trigeminal

nucleus caudalis of the cat (Goadsby & Knight, 1997; Mills & Martin, 1995).

Electrophysiological studies have shown that 5-HT_1 receptor agonists such as sumatriptan, dihydroergotamine and the new 5-$HT_{1B/D}$ receptor agonist rizatriptan are able to reduce the responsiveness of neurons in the C1/2 dorsolateral spinal cord of the cat and in the trigeminal caudal nucleus of the rat to electrical stimulation of the dura mater encephali (Cumberbatch *et al*., 1997; Hoskin *et al*., 1996; Kaube *et al*., 1993). Inhibition of synaptic transmission in the trigeminal nucleus could result either from a reduction of the release of transmitters and neuromodulators (presynaptic action) or from a reduction of excitability in postsynaptic neurons or both. Recent ionophoresis experiments in the cat have provided evidence that an inhibitory effect of 5-HT_1 receptor agonists is produced in the trigeminal caudal nucleus (Storer & Goadsby, 1997). The data of the present study suggests that a presynaptic action of CP 93,129 at this site could significantly contribute to the effect of CP 93,129. Moreover, in connection with the finding that a non-peptide NK-1 antagonist reduced the expression of c-*fos* in the subnucleus caudalis evoked by noxious chemical stimulation of the meninges (Cutrer *et al*., 1995), an important role of SP in the activation of neurons of the trigeminal nucleus is suggested.

These data, however, do not exclude that 5-HT_1 receptor agonists have also pronounced effects on the meningeal afferents in the periphery. Interestingly, we have found that the release of ir-SP was not reduced when CP 93,129 was administered topically to the brain stem. This finding suggests that the effect of intravenous CP 93,129 on the release of ir-SP was not due to an action in the brain stem itself. However, this conclusion has to be drawn with some caution for two reasons. Firstly, with the procedure of the experiment the time course of release in the vehicle-treated animals was somewhat different from that seen after intravenous injection. Maximum release of ir-SP was seen in the period 50–90 min after the stimulation. Thus the local application of a piece of cotton sucked with the vehicle or the vehicle plus compound may have altered the conditions or the measurement of ir-SP release. This must be particularly considered after the application of CP 93,129 which like other 5-HT_1 receptor agonists has some vasoconstrictory effect on dural vessels by activating vascular 5-HT_1 receptors (Messlinger *et al*., 1997). CP 93,129 might also act on the cerebral vessels of the brain stem. Secondly, it is not entirely clear which concentration of CP 93,129 reached the peptidergic afferent terminals in the spinal trigeminal nucleus after the 10 min of topical application, because the diffusion rates of CP 93,129 in neuronal tissues are not known. Nevertheless, the failure of CP 93,129 to reduce release after application to the brain stem is in marked contrast to the effectiveness of CP 93,129 after intravenous administration.

Our results suggest that 5-HT_1 receptor agonists decrease the excitability of primary meningeal afferents projecting to the spinal trigeminal nucleus, and this could result in reduction of both peripheral and central release of neuropeptides from the same afferents. There is evidence that the 5-HT_1 receptor agonist sumatriptan (which poorly penetrates the blood-brain barrier) is able to decrease the responses of second order neurons in the spinal trigeminal nucleus to mechanical and acidic stimulation of the dura mater (unpublished results from our laboratory). This finding also suggests that 5-HT_1 receptor agonists may block the afferent inflow to central trigeminal neurons by a peripheral rather than a central action.

In summary, the present data suggest that the activation of 5-HT_1 receptors reduces the release of SP from terminals of meningeal afferents in the spinal trigeminal nucleus, where SP seems to act as an important mediator in the nociceptive processing (Henry *et al*., 1980). This may be in particular relevant for the therapy of migraine pain and cluster headache, which can be alleviated by 5-HT_1 receptor agonists such as sumatriptan (Wilkinson *et al*., 1995). Although the site of action of these drugs can be located peripherally (including the trigeminal ganglion), they may cause a general decrease of neuropeptide release, the main benefit of which may be the reduced release and action of SP within the spinal trigeminal nucleus. This may also explain why NK-1 receptor antagonists with limited penetration through the blood-brain barrier are ineffective in the treatment of migraine attacks (Goldstein *et al*., 1997).

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