Objective.—To explore whether pharmacological stimulation of the 5-hydroxytryptamine 7 (5-HT7) receptor modulates Fos-like immunoreactivity in the trigeminal nucleus caudalis of rats.

Background.—The serotonin 5-HT7 receptor was proposed to be involved in migraine pathogenesis and evidence suggests it plays a role in peripheral nociception and hyperalgesia through an action on sensory afferent neurons.

Methods.—The potential activating or sensitizing role of 5-HT7 receptors on trigeminal sensory neurons, as visualized by Fos-like immunoreactivity in the superficial layers of the trigeminal nucleus caudalis in rats, was investigated using the 5-HT7 receptor agonist, LP-211, in the absence and the presence of intracisternal capsaicin, respectively. The agonist effect was characterized with the 5-HT7 receptor antagonist, SB-656104. Male Wistar rats received a subcutaneous injection of LP-211, SB-656104, and SB-656104 + LP-211. They were then anesthetized and prepared to receive an intracisternal injection of capsaicin or its vehicle. Animals were perfused and brains removed; sections of the brain stem from the area postrema to the CI level were obtained and processed for Fos immunohistochemistry.

Results.—Capsaicin but not its vehicle induced Fos-like immunoreactivity within laminae I and II of trigeminal nucleus caudalis. Pretreatment with LP-211 had no effect on Fos-like immunoreactivity but strongly increased the response produced by capsaicin; this effect was abolished by SB-656104. Interestingly, capsaicin-induced Fos-like immunoreactivity was abolished by SB-656104 pretreatment thus suggesting involvement of endogenous 5-HT.

Conclusions.—Data suggest that 5-HT7 receptors increase activation of meningeal trigeminovascular afferents and/or transmission of nociceptive information in the brain stem. This mechanism could be relevant in migraine and its prophylactic treatment.

Key words: 5-hydroxytryptamine 7, Fos-like immunoreactivity, trigeminal nucleus caudalis, capsaicin, cephalic pain, rat

Abbreviations: 5-HT 5-hydroxytryptamine, Fos-LI Fos-like immunoreactivity, i.e. intracisternal, LI-II laminae I and II, Sp5C trigeminal nucleus caudalis

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and transmitting zones of the brain stem. Immunoreactivity to the Fos protein, which is the product of the proto-oncogene c-fos, in the outer layers or laminae (LI-II) of Sp5C has been widely used as a marker of cephalic pain in experimental animals in response to chemical and mechanical stimulation of intracranial nerves. Thus, Fos-like immunoreactivity (Fos-LI) in Sp5C (LI-II) in response to aversive stimuli may be used to elucidate trigeminovascular mechanisms of neurovascular headaches.

Serotonin (5-hydroxytryptamine [5-HT]) has long been implicated in migraine and other vascular headaches but the nature of its involvement has not been defined. Regarding a potential ability of 5-HT to functionally modulate the trigeminovascular system, available studies have limited to the analysis of inhibitory mechanisms (5-HT1D/5-HT1F receptors) as potential targets of abortive treatments for migraine (eg, sumatriptan), but no studies have focused on the search for excitatory mechanisms that might play a role in the activation of this system. In this regard, it is interesting that 5-HT has actually been demonstrated to play a role in pain and hyperalgesia in both humans and experimental animals, thus implying that the monoamine may indeed play an excitatory role in nociceptive pathways. Among the numerous 5-HT receptor (sub)types classified to date, evidence has become available to suggest that the 5-HT7 receptor is involved in pain and hyperalgesia by mechanisms that may involve neuronal excitation. Whether the 5-HT7 receptor might also be present in the trigeminovascular system and play a role in cephalic pain and hyperalgesia is not known. The reported expression of the 5-HT7 mRNA in human trigeminal ganglia, however, seems consistent with this possibility.

On the basis of the above it was therefore hypothesized that pharmacological activation of the 5-HT7 receptor could possibly lead to activation of trigeminovascular afferents supplying the meninges, as reflected by an increase of Fos-LI in Sp5C (LI-II). Furthermore, it has been shown that the cyclic adenosine monophosphate/protein kinase A second messenger cascade is implicated in hyperalgesia induced by inflammatory mediators and that this pathway mediates sensitization of dural nociceptive neurons. As this signaling pathway represents the major transduction mechanism of 5-HT7 receptors, it was also hypothesized that 5-HT7 receptor stimulation could promote sensitization of trigeminovascular sensory fibers to the activating action of capsaicin. The aim of the present study in rats was therefore to evaluate the effect of a selective 5-HT7 receptor agonist on Fos-LI in Sp5C (LI-II) in the absence and the presence of intracisternal (i.c.) capsaicin, and characterize this effect by using a 5-HT7 receptor antagonist. The results suggest that 5-HT7 receptors may play a modulatory role in the trigeminovascular system by increasing capsaicin-induced activation of trigemovascular sensory fibers. A preliminary account of this investigation has been presented in abstract form.

METHODS

Animals.—Male Wistar rats (250-300 g; n = 49) were purchase from Harlan Mexico (Mexico City). The animals were individually housed for at least 3 days before the experiments and kept at constant temperature and a 12-hour light : dark cycle with food and water provided at libitum. Efforts were made to restrict the number of animals used and to avoid any unnecessary suffering of them. All procedures and protocols complied with federal regulations on the care and use of laboratory animals and were approved by the Cinvestav-IPN ethics committee (CICUAL).

Intracisternal Injection of Capsaicin.—After anesthesia with sodium pentobarbital (50 mg/kg i.p.), the animals were placed in a stereotaxic frame and prepared to receive an i.c. injection of capsaicin. For this purpose, a midline skin incision was made from the occipital protuberance to the cervical area under semi-sterile conditions and a 27-gauge needle coupled to a Hamilton syringe was inserted into the cisterna magna; the correct position of the needle in the cisterna magna was confirmed by extracting a small amount of cerebrospinal fluid. During this procedure, animals were mechanically ventilated through the trachea with a Harvard Apparatus rodent ventilator (58 cycles/min; 2 mL/100 g body weight). Capsaicin was administered with the aid of a
micro-injection pump at a rate of 20 μL/min during 5 minutes for a total volume of 100 μL; this 100 μL i.c. injection has been widely used by other research groups with the vehicle shown to be devoid of remarkable effects on background levels of Fos-LI in Sp5C. 26, 27 To prevent capsaicin leakage, the needle was removed 10 minutes after the injection was completed. In order to facilitate appropriate distribution of capsaicin, the animals were kept in a reverse Trendelenburg position for 30 minutes and then in a lateral position for 90 minutes. Control animals received an i.c. injection of vehicle. Evans Blue (0.2%) staining of the epidural space was used to demarcate the distribution of capsaicin in the skull; the dye was co-injected with capsaicin or its vehicle. The staining pattern, which was used to verify the effectiveness of the procedure, exhibited the typical pattern reported in other studies with a stronger coloration in the dura mater ventral from the cerebellum, around the brain stem and the upper spinal cord; 28 the animals that did not show this pattern were excluded from the study. The body temperature of the animals was maintained at 37 ± 1°C with a thermostatically regulated homeothermic blanket and anesthesia was maintained by supplemental injections of pentobarbital (10 mg/kg, i.p). At the end of the 2-hour period animals were perfused via the ascending aorta with 0.1 M phosphate-buffered saline (PBS) at pH 7.4 for 6 minutes, and 4% paraformaldehyde in 0.1 M PBS for 30 minutes. The brain stem with attached cervical cord was stored overnight in the same fixative and then placed in a cryoprotectant (30% sucrose in 0.1 M PBS) for 48 hours. Serial coronal sections (40 μM thick) were prepared at −20°C with a SM2000R Leica freezing microtome; sections were taken from the area postrema to the C1 level with every third section being saved and processed for immunohistochemistry.

**Fos Immunohistochemistry.**—Induction of the Fos protein was characterized immunohistochemically in free floating sections with the avidin-biotin procedure using commercially available kits. Sections were rinsed in 0.1 M PBS, pretreated with 2% bovine serum albumin and 1% hydrogen peroxide in 0.1 M PBS for 2 hours at room temperature. They were then incubated with a primary rabbit polyclonal Fos antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 0.1 M PBS with 0.3% Triton X-100 for 24 hours; after this period sections were rinsed and placed in 0.1 M PBS with 0.02% Triton X-100 containing biotinylated goat anti-rabbit (1:100) secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 2 hours. After several washes in 0.1 M PBS with 0.02% Triton X-100, the sections were incubated with an avidin-biotin-peroxidase complex (VECTASTAIN Elite ABC kit, 1:200; Vector Laboratories) in 0.1 M PBS with 0.3% Triton X-100 containing Tris-buffer, 3,3′-diaminobenzidine, hydrogen peroxide and nickel in distilled water. Sections were then washed, mounted on gelatin-coated slides and cover-slipped. All procedures were performed under gentle tridimensional agitation.

**Fos-Immunoreactive Cell Counting.**—All Fos-immunoreactive cells were counted bilaterally in LI-II of Sp5C in 7 sections from each animal containing the trigeminal nucleus from the area postrema to the C1 level. Then, the mean number of labeled cells per section was calculated for each animal and then an average number for each animal group was obtained.

**Experimental Protocol.**—Rats were divided into 8 experimental groups that received the following treatments: (1) vehicle for capsaicin (n = 6); (2) capsaicin (1 μM) (n = 7); (3) LP-211 (10 mg/kg) + vehicle for capsaicin (n = 6); (4) LP-211 (10 mg/kg) + capsaicin (1 μM) (n = 6); (5) SB-656104 (3 mg/kg) + vehicle for capsaicin (n = 6); (6) SB-656104 (3 mg/kg) + capsaicin (1 μM) (n = 6); (7) SB-656104 (3 mg/kg) + LP-211 (10 mg/kg) + vehicle for capsaicin (n = 6); and (8) SB-656104 (3 mg/kg) + LP-211 (10 mg/kg) + capsaicin (1 μM) (n = 6). Exception made of capsaicin and its vehicle, which were injected into the cisterna magna, all the other treatments were given subcutaneously between 20 minutes (LP-211) and 40 minutes (SB-656104) before the animals were anesthetized and prepared to receive the i.c. injection. The s.c. route was preferred.
over the i.p. route in order to maximally reduce stressful manipulation of the animals.

Drugs.—Apart from the anesthetic, the drugs used in the present study (obtained from the sources indicated) were the following: capsaicin (8-methyl-N-vanillyl-6-nonenamide; Tocris); LP-211 (N-(4-cyanophenylmethyl)-4-(2-diphenyl)-1-piperazinexanamide) (Dr. Marcello Leopoldo, Università degli Studi di Bari, Bari, Italy); and SB-656104 (Gift from Glaxo-SmithKline, Harlow, UK). Capsaicin (3.05 mg) was diluted in 1 mL of saline : ethanol : Tween 80 (8:1:1) and sonicated for 30 minutes. This stock solution (10 mM) was diluted to a final 1 μM concentration (305 ng/mL) of capsaicin. Solutions of LP-211 and SB-656104 were prepared by dissolving the drugs in 20% dimethyl sulfoxide in distilled water; this vehicle did not alter Fos-LI in Sp5C (LI-II) (data not shown). Fresh solutions were prepared for each experiment.

Data Presentation and Statistical Analysis.—The number of Fos-immunoreactive cells per treatment group is expressed as the mean per section ± SEM. Comparison between the different group treatments was performed with one-way analysis of variance followed by a Newman-Keuls test to determine differences using the statistical tools of GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). In all cases, statistical significance was accepted at P < .05 (two-tailed).

RESULTS

Consistent with previous findings in this model,26-29 i.c. injection of 1 μM capsaicin—but not of its vehicle—(Fig. 1) significantly increased Fos-LI in Sp5C (LI-II). Fresh solutions were prepared for each experiment.

Fig 1.—Distribution of Fos-immunoreactive cells in Sp5C (LI-II). Treatments were as follows: vehicle (i.c.) (A,B); capsaicin (1 μM, i.c.) (C,D); LP-211 (10 mg/kg, s.c.) + capsaicin (1 μM, i.c.) (E,F); and SB656104 (3 mg/kg, s.c.) + LP-211 (10 mg/kg, s.c.) + capsaicin (1 μM, i.c.) (G,H). Upper and lower panels show, respectively, 10× and 20× microphotographs of the same preparation. Inserts in upper panels demarcate the areas shown in the lower panel microphotographs. Bars = 200 μM (upper panels) and 100 μM (lower panels).
Sp5C (LI-II) (Fig. 2). Capsaicin-induced Fos-LI was observed in clusters that were located predominantly in the dorsal and ventral aspects of Sp5C and they were present bilaterally. Subcutaneous administration of the selective 5-HT 7 receptor agonist, LP-211 (10 mg/kg), followed by i.c. injection of capsaicin’s vehicle did not change Fos-LI in Sp5C (LI-II) as compared with the effect of the vehicle alone; a trend towards an increase, however, was noted (values for capsaicin’s vehicle alone and LP-211 + capsaicin were, respectively, 42 ± 18 and 77 ± 24 immunopositive cells per section) (Fig. 2). Interestingly, notwithstanding, pretreatment of the animals with LP-211 strongly and significantly increased Fos-LI induced by i.c. capsaicin (values for capsaicin alone and LP-211 + capsaicin were, respectively, 164 ± 29 and 55 ± 11 immunopositive cells), thereby suggesting involvement of endogenous 5-HT in the effect of the irritant (Fig. 2).

**DISCUSSION**

The major finding of the present study is that the 5-HT 7 receptor agonist, LP-211, given at a dose consistent with specific stimulation of 5-HT 7 receptors in vivo, strongly increased Fos-LI induced by i.c. capsaicin in LI-II of Sp5C. This result seems remarkable as it represents an evidence for an excitatory serotonergic mechanism in the trigeminovascular system, which might play a role in the pathogenesis of migraine, as hypothesized previously. The 10 mg/kg dose of LP-211 was selected as this was previously demonstrated to rapidly reach the systemic circulation in mice achieving a mean C max of 0.76 ± 0.32 μg/mL at 30 minutes with quantifiable levels remaining evident for a period of 2 hours, and produce a significant hypothermic effect in wild type but not in 5-HT 7 receptor knockout mice, with the lower 3 mg/kg i.p. dose being inactive. Although the 30 mg/kg i.p. dose of LP-211 was reported to produce a higher hypothermic effect in wild type mice as compared with the 10 mg/kg dose, it also induced hypothermia in 5-HT 7 receptor knockout mice; furthermore, the hypothermic response to the 30 mg/kg dose of LP-211 in wild type mice, in addition to being blocked by the 5-HT 7 receptor antagonist, WAY-100635, thus suggesting interaction of LP-211 with other receptor mechanisms at this higher dose. For these reasons, and because of the restricted number of animals to be used in the study, it was decided to limit the use of LP-211 to the 10 mg/kg dose, which, on the basis of the aforementioned studies, appears to selectively stimulate 5-HT 7 receptor.
receptors. Regarding the 1-arylpiperazine metabolite of LP-211, which is more potent and selective than LP-211 for 5-HT7 receptors, its plasma concentrations were shown to be below the detection limit of the analytical procedure when administered at the 3 mg/kg and 10 mg/kg doses in mice; it seems therefore unlikely that LP-211’s metabolite may have played a significant role in the effects produced by the 10 mg/kg dose of the parent compound on Fos-LI. In spite of the fact that rats were used in the present experiments and that no LP-211 disposition studies have been conducted in this species, it was assumed that this dose of the drug would most likely result in significant activation of 5-HT7 receptors over a period of 2 hours after administration. Mediation by 5-HT7 receptors in the above effect of LP-211 on capsaicin-induced Fos-LI was confirmed by the ability of the 5-HT7 receptor antagonist, SB-656104, to completely inhibit this response. The 3 mg/kg s.c. dose of SB-656104 was chosen to ensure consistent blockade of 5-HT7 receptors during the entire course of the experiment as an ED50 oral dose of 2 mg/kg of the compound was previously determined to antagonize 5-CT-induced hypothermia in guinea pigs with a maximal inhibition being observed 2 hours after administration. In agreement with this observation, a 3 mg/kg i.p. dose of SB-656104 given 1 hour before was recently reported to reverse the learning impairment induced by the N-methyl-D-aspartate receptor antagonist, MK-801, in the Morris water maze test in rats. Since in the present experiments SB-656104 (3 mg/kg s.c.) was administered 40 minutes before i.c. treatments, the compound was clearly within a time frame consistent with its ability to block central and peripheral 5-HT7 receptors. This antagonist has been shown to exhibit reasonable 5-HT7 receptor selectivity with over 12-, 31-, 45- and 91-fold higher affinity for the 5-HT7 receptor (pKi = 8.7) with respect to 5-HT3D (pKi = 7.6), 5-HT2A (pKi = 7.2), 5-HT2B (pKi = 7.04), and 5-HT3A receptors (pKi = 6.74), respectively.

Induction of the product of the immediate early gene c-fos is accepted to reflect functional activity in neurons, and immunoreactivity to the Fos protein in LI-II of Sp5C has been used by a number of research groups to study the activity of the sensory part of the trigeminal system. Thus, it is likely that 5-HT7 receptor activation might be involved in headache conditions that result from activation of the trigeminovascular system including migraine and cluster headache. This notion is consistent with a growing body of evidence supporting a role of 5-HT7 receptors in nociception and hyperalgesia. Indeed, the 5-HT7 receptor mRNA was reported in sensory nerve structures such as the dorsal root ganglia of humans and rats, whereas the protein was both detected in primary afferent nociceptors (which terminate in the superficial layers I and II of the spinal cord dorsal horn) and demonstrated to be involved in 5-HT7-induced nociceptor activation. Relevant to the present findings and the potential involvement of the 5-HT7 receptor in mechanisms of migraine headaches, evidence was provided suggesting that 5-HT7 receptors might increase capsaicin-induced neurogenic inflammation in the rat knee joint.

Whether the 5-HT7 receptor is expressed in the trigeminovascular system is not known at the present time, but its mRNA was found in human trigeminal ganglia. As trigeminal nuclei in the medullary brain stem constitute an anatomical continuum of the spinal cord, it seems reasonable to expect that 5-HT7 receptors may also be expressed and play a nociceptive role in this area, which is highly relevant in the transmission of nociceptive information from meningeal tissues via trigeminal nociceptive fibers. The present data do not allow us to establish whether 5-HT7 receptors mediating the amplifying effect of LP-211 on capsaicin-induced Fos-LI in Sp5C are expressed on peripheral trigeminovascular afferents and/or second-order neurons within the brain stem (see further discussion). Further studies will be required to precisely locate 5-HT7 receptors in the trigeminovascular system. It should be emphasized on the other hand that, as the single administration of LP-211 had no effect on Fos-LI with respect to controls, it is unlikely that additional direct/indirect peripheral or central effects of this brain penetrant agonist had played a significant role on capsaicin-induced Fos-LI in Sp5C.

It is finally interesting from a pathogenic standpoint that capsaicin-induced Fos-LI was abolished by
SB-656104, which suggests that capsaicin-induced activation of trigeminovascular afferents was mediated by release of endogenous 5-HT (acting on 5-HT<sub>7</sub> receptors). It has been shown in this connection that a plausible source of 5-HT in the trigeminovascular system is the dural mast cell population, as electrical stimulation of the trigeminal ganglion, leading to activation of meningeal nociceptors, promotes degranulation of mast cells in the dura mater. Such activation of meningeal nociceptors has been hypothesized to take place during migraine with aura in response to local release of protons, potassium ions and glutamate. Activated meningeal nociceptors appear to release neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) that induce activation and degranulation of resident dural mast cells. The local release of inflammatory molecules from degranulated mast cells during neurogenic inflammation is believed to further stimulate meningeal nociceptors to promote a prolonged migraine headache. The ability of the 5-HT<sub>7</sub> receptor to amplify capsaicin-induced Fos-LI in Sp5C (LI-II) (present results) is actually consistent with this notion, thus raising the possibility that the effect of 5-HT<sub>7</sub> receptor stimulation by 5-HT could involve CGRP release from trigeminovascular afferents with both substances acting synergistically to exacerbate activation of these fibers. In further support of this possibility, it was preliminarily found that topical application of LP-211 on the dura mater encephali increases blood flow in the middle meningeal artery of anesthetized rats via a 5-HT<sub>7</sub> receptor-mediated mechanism that involves CGRP release; this functional evidence would certainly support the location of 5-HT<sub>7</sub> receptors in meningeal trigeminovascular afferents.

In conclusion, the present study has provided evidence for an excitatory serotonergic mechanism mediating facilitation of capsaicin-induced Fos-LI in Sp5C (LI-II) via activation of 5-HT<sub>7</sub> receptors probably located in trigeminovascular afferents. In support of this possibility, during the preparation of this manuscript a report was published on the ability of the 5-HT<sub>7</sub> receptor antagonist, AS-19, failed to alter electrically-induced CGRP release upon single administration but it, however, reversed the inhibitory effect of SB-269970 on CGRP release. Activation of 5-HT<sub>7</sub> receptors by 5-HT (probably released from activated dural mast cells) and their blockade by selective 5-HT<sub>7</sub> receptor antagonists could therefore be, respectively, relevant mechanisms in the pathogenesis and prophylactic treatment of migraine headaches.

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