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Neuropeptide release from slices of rat and guinea pig trigeminal ganglia: modulation by dihydroergotamine and sumatriptan

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Abstract The trigeminovascular system is considered to play a role in the mechanism of migraine headache. Novel in vitro animal models that investigate the release of neuropeptides may be of help to understand the pathophysiology and pharmacology of trigeminal neurons. Here, we examined the release of the immunoreactivity (LI) of the sensory neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP) from slices of rat and guinea pig trigeminal ganglia with proximal nerve trunks attached. Electrical field stimulation (EFS, 10 Hz), high K^+ medium (50 mM) and capsaicin (1 μM) caused a significant increase in CGRP-LI outflow. SP-LI was also released after exposure to EFS, high K^+ and capsaicin. The increase in CGRP-LI outflow induced by EFS was markedly reduced in a Ca^{2+}-free medium and by pretreatment with a high capsaicin concentration, tetrodotoxin, ω-conotoxin, dihydroergotamine and sumatriptan. Sensory neuropeptide release from slices of rat trigeminal ganglia with nerve trunks attached fulfills the criteria required to define it as a neurosecretory event. This is a novel method for studying trigeminal neuron pathophysiology and the action of antimigraine drugs.

Key words Substrance P • Calcitonin gene-related peptide • Sensory neurons • Neurogenic inflammation • Migraine

Introduction

Despite the recent improvement in the acute treatment of migraine headache attacks [1], the pathophysiological mechanism of this disease is still obscure. This, at least in part, results from the absence of reliable models of this disorder in experimental animals. In the past 15 years major attention has been focused on the role of the trigeminovascular system as the anatomical site where symptoms originate during the migraine attack [2, 3]. Trigeminal ganglion neurons, considered to be important for migraine headache, consist of a subpopulation of primary sensory neurons that contain neuropeptides, such as the tachykinins, substance P (SP) and neurokinin A (NKA) [4], and the calcitonin gene-related peptide (CGRP) [5]. Tachykinins and CGRP are transported to and released from peripheral and central endings of primary sensory neurons [4]. The release of neu-

ropeptides from peripheral terminals of primary sensory neurons causes a series of inflammatory responses that are collectively referred to as "neurogenic inflammation" [6]. Peripherally released SP and NKA via NK_1 receptor activation open up gaps between endothelial cells in postcapillary venules, thus causing extravasation of plasma proteins [7, 8]. The prevalent action of CGRP, via the activation of CGRP receptors and probably modulated by the recently discovered RAMPs [9], is arterial dilatation [10]. At the central level, release of SP has been associated with pain transmission [11, 12]. Thus, sensory neuropeptides appear to be involved in all the main features of the migraine attacks, including vasodilatation, perivascular edema and pain.

The hypothesis that peptidergic trigeminal neurons play a role in migraine headache has been reinforced by the observation that the antimigraine drug sumatriptan reduces both the release of SP and CGRP [13] and their effects on cephalic target tissues [14] by stimulating inhibitory 5-$HT_{1B/D}$ receptors [15]. There is evidence that CGRP-like immunoreactivity (CGRP-LI) is increased in plasma taken from the jugular vein during migraine [16] and cluster headache [17] attacks, and that the release is reduced after the administration of sumatriptan. This observation underlines the potential of methods that allow the study of sensory neuropeptide release from trigeminal primary sensory neurons and its pharmacological modulation.

The aim of the present study was to examine whether different stimuli could elicit the release of CGRP-LI from thick (0.4 mm) slices of freshly dissociated rat trigeminal ganglia with proximal nerve trunks attached. We also studied the physiological and pharmacological characteristics of the CGRP-LI release from this preparation in order to determine whether this method could be suitable for the in vitro study of the pharmacodynamic properties of potential antimigraine drugs. The data show that CGRP-LI release from rat trigeminal ganglia with proximal nerve trunks attached fulfills the criteria required to define it as a neurosecretory event. In a few experiments the release of the sensory neuropeptide SP was also studied. In order to demonstrate that the present findings were not species-specific, CGRP-LI release from slices of guinea pig trigeminal ganglia with the nerve trunks attached was also studied.

Materials and methods

Animals and tissues

Male albino rats (Wistar, 250–300 g) and guinea pigs (Dunkin-Hartley strain, 240–280 g) (Charles-River, Italy) were used. Rodents were sacrificed with pentobarbital (60 mg/kg intraperitoneally). The skull was open, the brain gently removed and the trigeminal ganglion exposed. Nerve trunks were cut and the ganglion was quickly transferred to an oxygenated 96% O_2, 4% CO_2 modified Krebs solution (119 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $KHPO_4$, 1.5 mM $MgSO_4$, 4.7 mM KCl, 2.5 mM $CaCl_2$ and 11 mM glucose) maintained at 4° C.

Slices of trigeminal ganglia (0.4 mm) were prepared at 4° C using a tissue slicer (Stoelting, Chicago, Illinois). Slices (50–80 mg) were placed in 2 ml chambers and superfused at 0.4 ml/min with Krebs solution containing 0.1% bovine serum albumin, 1 μM phosphoramidon and 1 μM captopril at 37° C and gassed with 96% O_2, 4% CO_2. After a 60-min stabilization period, 5-min fractions were collected into acetic acid (final concentration, 2 M). Electrical stimuli (10 Hz, 1 ms pulse duration, 100 mA/cm^2, for 5 min) were administered with platinum electrodes connected to a Grass S88 stimulator. When high K^+ was used, the appropriate amount of NaCl was isotonically replaced by KCl. In some experiments, tissues were pre-incubated for 20 min with 10 μM capsaicin 90 min prior to the administration of the stimulus, or tissues were, perfused in Ca^{2+}-free medium containing 1 mM EDTA. At the end of the experiment tissues were blotted and weighed. Fractions were freeze-dried, reconstituted with assay buffer and analyzed by enzyme immunoassays for CGRP and SP, respectively.

CGRP-LI and SP-LI assays

CGRP-LI was measured by a sensitive sandwich (two-sites) enzyme immunoassay, which uses the mAb CGRP-83 as the capture antibody. The mAb CGRP-72, covalently labeled with enzyme acethylcholinesterase, acts as tracer. The detection limit of the assay was 2 pg/ml [18]. The assay has been used to determine specific concentrations of CGRP in different species, including rat, mice and guinea-pig samples [18]. Cross-reactivity with calcitonin was < 0.01%. SP-LI was measured by using a conventional competitive enzyme immunoassay in which SP-acetylcholinesterase conjugate was used as tracer [19]. The detection limit of the assay was 2 pg/ml. Cross-reactivity with NKA was < 0.01%. Results are expressed as fmol peptide/g tissue per 10 minutes.

Reagents

SP and CGRP ω-conotoxin were from Peninsula (Merseyside, UK). Dihydroergotamine, tetrodotoxin and capsaicin were from Sigma (St. Louis, MO). Sumatriptan was a kind gift from P.A.A. Humphrey (University of Cambridge). Capsaicin was diluted in ethanol (10 mM); further dilutions were performed in Krebs solution.

Statistical analysis

All data in the text and figures are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by analysis of variance and Dunnett's test for multiple comparisons when appropriate.

Results

Rat trigeminal ganglia

Baseline CGRP-LI in the effluent from slices of rat trigeminal ganglia with the nerve trunks attached was 1.2 ± 0.2 (n = 12). Exposure to EFS for 5 min caused an increase of more than 8-fold in the outflow of CGRP-LI. Similarly, exposure to high K^+ (50 mM for 10 min) caused a significant increase in CGRP-LI outflow. Finally, capsaicin (1 μM for 10 min) caused a remarkable increase (more than 20-fold) in the CGRP-LI outflow (Fig. 1).

A second administration of EFS (S2) for 5 min (90 min after the first administration) caused an increase in CGRP-LI that was similar to that obtained with the first administration of EFS (S1) (Fig. 2a). The S2/S1 ratio was 0.81 ± 0.06 (n = 6). The S2/S1 ratios reported in Fig. 2b indicate that after pre-exposure to a high (desensitizing) capsaicin concentration

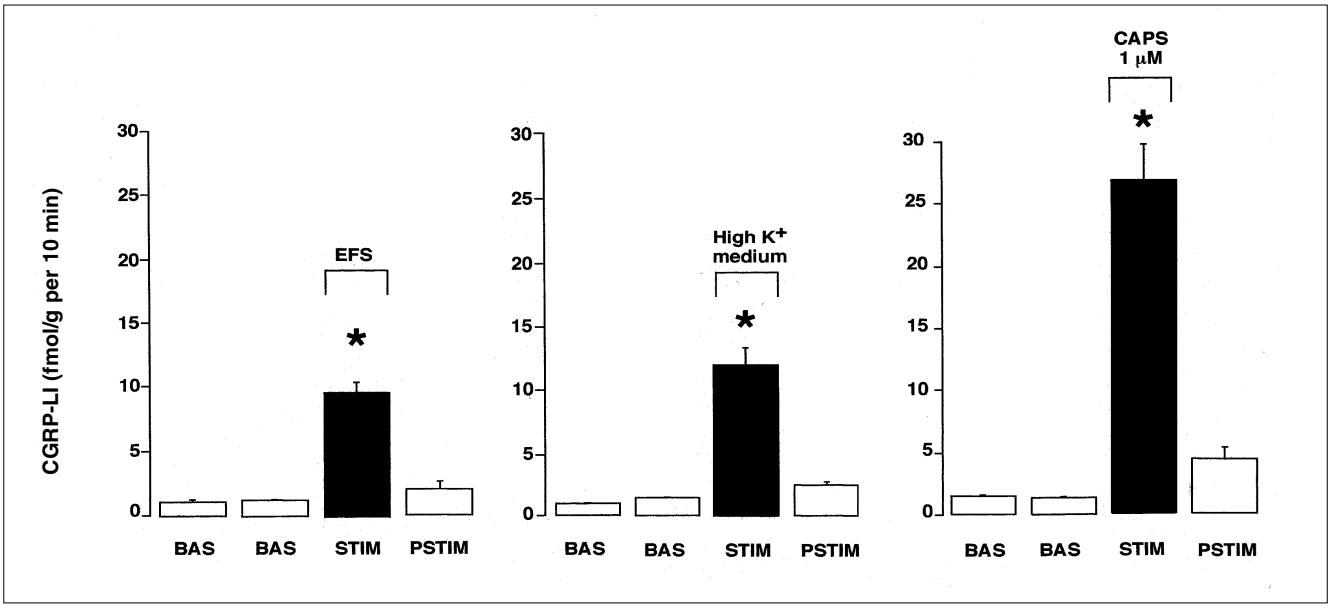

Fig. 1 CGRP-LI outflow induced by electrical field stimulation (*EFS*, 10 Hz), high K^+ medium, or capsaicin (*CAPS*) from slices of rat trigeminal ganglion with nerve trunks attached. Each column represents a 10-min fraction and is the mean of 6 experiments. *BAS*, baseline; *STIM*, stimulus; *PSTIM*, post-stimulus. * $p < 0.05$ vs. first baseline value

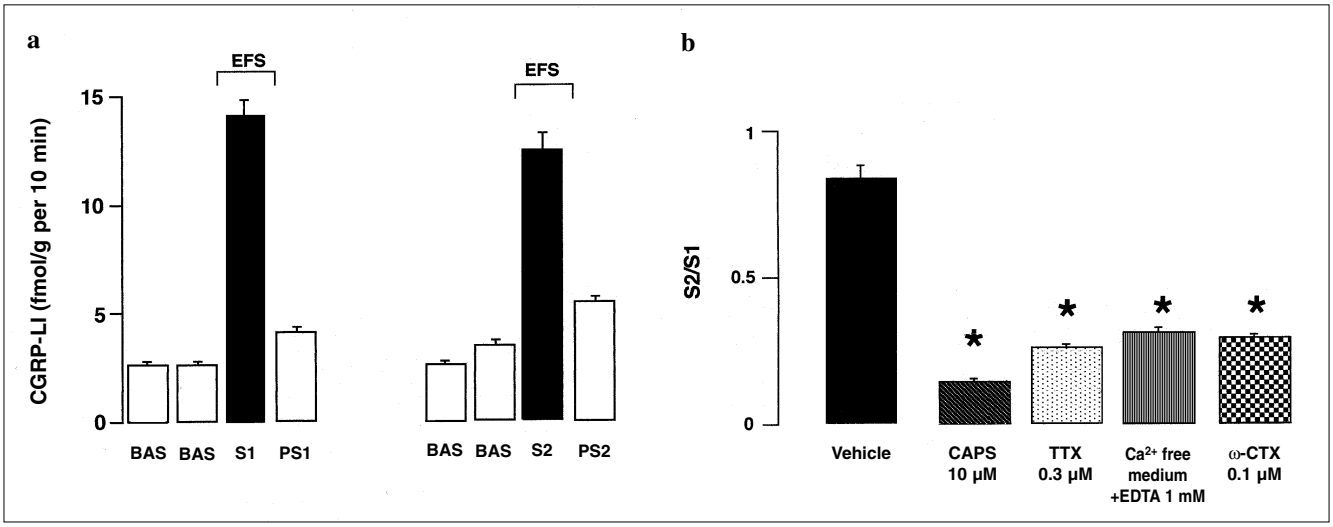

Fig. 2 a CGRP-LI outflow induced by a first (*S1*) and a second (*S2*, 90 min after the first administration) administration of electrical field stimulation (*EFS*, 10 Hz) from slices of rat trigeminal ganglion with nerve trunks attached. *BAS,* baseline; *S1* and *S2,* electrical stimulation; *PS1* and *PS2*, post-stimulus. **b** Effect of capsaicin (10 μM for 20 min) pre-treatment, tetrodotoxin (TTX), ω-conotoxin (CTX) and Ca^{2+}-free medium on CGRP-LI outflow induced by EFS (10 Hz) from slices of rat trigeminal ganglion with nerve trunks attached. Values are expressed as S2/S1 ratio. Each column is the mean of at least 4 experiments. * $p < 0.05$ vs. vehicle

[20], the S2/S1 ratio was reduced by 83%, and in a Ca^{2+}-free medium plus EDTA (1 mM) it was inhibited by 64%. In the presence of the fast sodium channel blocker, TTX, and the N-type Ca^{2+}-channel inhibitor, ω-conotoxin, the S2/S1 ratio was significantly reduced by 70% and 65%, respectively.

In the presence of the antimigraine drugs, dihydroergotamine (1 μM) or sumatriptan (1 μM), baseline CGRP-LI outflow was 0.9 ± 0.2 fmol/g per 10 min (n = 5) and 1.1 ± 0.1 fmol/g per 10 min (n = 5), respectively. These values were not different from baseline values obtained in the presence of dihydroergotamine or sumatriptan vehicles (data not shown). In the presence of dihydroergotamine (1 μM) or sumatriptan (1 μM), the S2/S1 ratio was also reduced by 65% and 68%, respectively (Fig. 3a). In another set of experiments run in parallel, the presence of dihydroergotamine (1 μM) or sumatriptan (1 μM) reduced the increase in CGRP-LI outflow induced by 50 mM K^+ by 67% and 63%, respectively (Fig. 3b). In these experiments, baseline values in the presence of dihydroergotamine (1 μM) or sumatriptan (1 μM) or their vehicles were 0.8 ± 0.2 fmol/g per 10 min (n = 4), 1.2 ± 0.2 fmol/g per 10 min (n = 4) and 01.0 ± 0.1 fmol/g per 10 min (n = 6), respectively.

The outflow of SP-LI above baseline from slices of rat trigeminal ganglia with the nerve trunks attached was significantly increased by the administration of EFS, 50 mM K^+ and 1 μM capsaicin (Fig. 4).

Guinea-pig trigeminal ganglia

Administration of EFS for 5 min to slices of guinea pig trigeminal ganglia with the nerve trunks attached induced a significant increase in CGRP-LI outflow (Fig. 5).

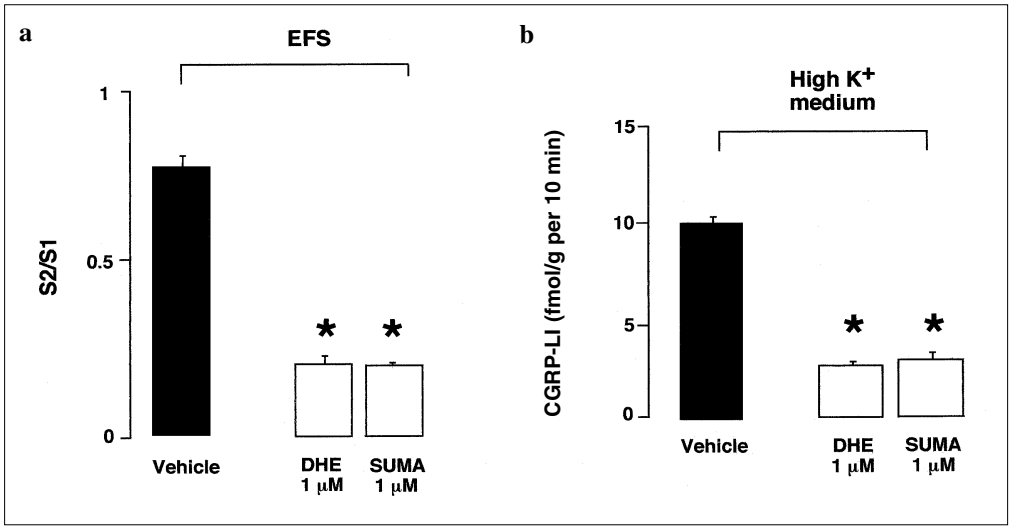

Fig. 3 Effect of dihydroergotamine (*DHE*) or sumatriptan (*SUMA*) on the increase in CGRP-LI outflow induced by electrical field stimulation (EFS, 10 Hz, **a** or high K^+ medium **b** from rat trigeminal ganglion with nerve trunks attached. Each column is the mean of at least 4 experiments. * $p < 0.05$ vs. vehicle

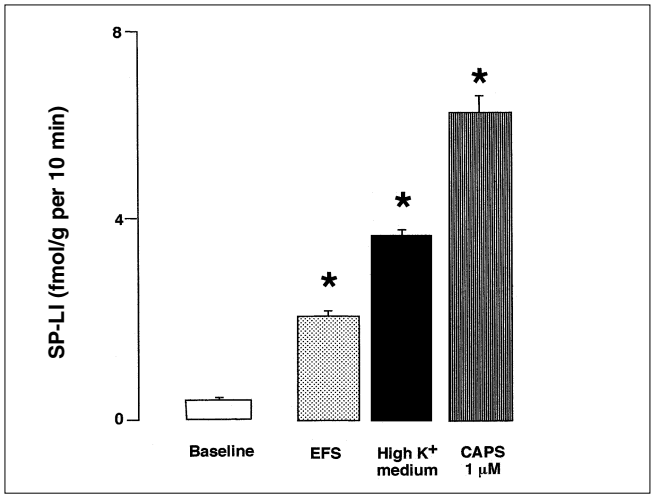

Fig. 4 Increase in SP-LI outflow from rat trigeminal ganglion with nerve trunks attached induced by EFS (10 Hz), high K^+ medium, or capsaicin (*CAPS*). Each column is the mean of at least 4 experiments. * $p < 0.05$ vs. first baseline value

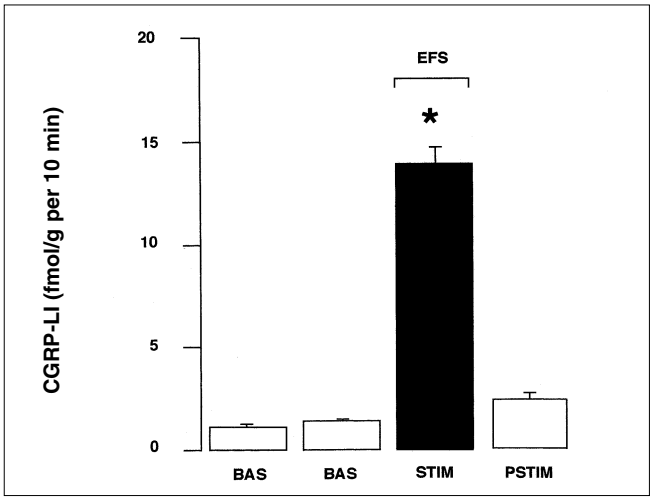

Fig. 5 Increase in CGRP-LI outflow induced by electrical field stimulation (EFS, 10 Hz), from slices of guinea pig trigeminal ganglion with nerve trunks attached. *BAS*, baseline; *STIM*, stimulus; *PSTIM*, post-stimulus

Discussion

The results presented in this study show that the increase in CGRP-LI outflow induced by EFS fulfills the criteria needed to define this as a neurosecretory event that originates from capsaicin-sensitive primary sensory neurons. First, an increase in CGRP-LI induced by this stimulus was practically abolished when experiments were performed in a Ca^{2+}-free medium, plus EDTA. Secondly, after exposure to a high (desensitizing) [20] concentration of capsaicin, EFS failed to evoke a further increase in CGRP-LI outflow. Thus, the observation that an increase in CGRP-LI level in the effluent from slices of rat trigeminal ganglia is Ca^{2+}-dependent and capsaicin-sensitive strongly supports the view that EFS causes neurosecretion of CGRP from capsaicin-sensitive primary sensory neurons of rat trigeminal ganglia. Additional support to this hypothesis is given by the observation that the increase in CGRP-LI outflow induced by EFS was completely abolished by TTX and by ω-conotoxin. This finding indicates that the applied depolarizing electrical stimulus evoked neuropeptide release by operating the fast Na^+ channels sensitive to TTX and the voltage-sensitive Ca^{2+}-channels of the N type [21].

The region of primary sensory neurons where neurotransmitter release is considered to occur physiologically is the nerve varicosity where, under adequate stimulation, vesicles of different sizes fuse to the plasma membrane and discharge their content into the synaptic space. This usually happens in the terminal region of the nerve fiber or a region somehow distal from the neuronal cell body. In this study, the preparations contained neuronal cell bodies and nerve trunks proximal to the cell body. The observation that capsaicin, EFS and high K^+ caused release of CGRP from this preparation suggests that cells bodies with their proximal nerve trunks attached express all the functional machinery required for the neurosecretory process of peptide transmitters. In the present case this consists of the recently cloned non-selective ion channel (VR-1) [22] operated by capsaicin, the TTX-sensitive fast sodium channel, and the ω-conotoxin sensitive voltage-dependent Ca^{2+}-channel. Our results also imply that vesicles containing neuropeptides are present in the cell bodies and proximal nerve trunks of capsaicin-sensitive, trigeminal primary sensory neurons, and that these vesicles are available for the neurosecretory process.

The observation that SP-LI outflow increased after exposure to EFS, high K^+ medium and capsaicin suggests that the ability of slices of rat trigeminal ganglia to release neuropeptides is not limited to CGRP, but involves additional sensory neuropeptides. In the present study we did not investigate the release of NKA. However, it is likely that our present experimental settings would have allowed detection of the increase of this additional sensory neuropeptides.

Most studies that have clarified the role of capsaicin-sensitive primary sensory neurons in nociceptive transmission and neurogenic inflammation have been performed in rats. However, data from a number of studies has also been obtained in other rodents, including guinea pigs [23]. We have tested the hypothesis that what has been demonstrated in rats is also applicable to guinea pigs. Findings support this hypothesis, as EFS was capable of increasing the outflow of CGRP-LI from slices of guinea-pig trigeminal ganglia with the nerve trunks attached. Thus, it is possible to advance the hypothesis that slices of guinea-pig trigeminal ganglia may be a suitable model to investigate mechanisms of sensory neuropeptide release and their pharmacological modulation.

The first part of this study gives support towards the second part in which the ability of drugs, commonly used in the treatment of the acute attack of migraine headache, to modulate the release of CGRP-LI was examined. Dihydroergotamine is far from being a selective drug, and it shares with ergotamine the ability to stimulate α-adrenoceptors, dopamine receptors and 5-HT receptors [24]. Among this pleiotropic range of actions, it may also bind with high affinity and stimulate $5\text{-}HT_{1B/D}$ receptor subtypes [13, 25]. In contrast sumatriptan, the first successful compound of this expanding class of antimigraine drugs, has a rather discriminating spectrum of action, because it stimulates selectively $5\text{-}HT_{1B/D}$ receptors [1]. This pharmacological property is considered the cause of its powerful antimigraine action. Stimulation of $5\text{-}HT_{1B/D}$ receptors, functionally, should result in the vasoconstriction of a variety of arterial vessels including those of the cephalic circulation, a phenomenon that is considered beneficial during the migraine attack [26, 27]. An alternative explanation of the antimigraine effect of sumatriptan relies on the ability of prejunctional $5\text{-}HT_{1B/D}$ receptors to inhibit the release of sensory neuropeptides from trigemimal primary sensory neurons, thus limiting neurogenic inflammation and nociceptive transmission [1, 3]. This hypothesis has found support from a number of functional and neurochemical studies in experimental animals. Additional proof for a role of sumatriptan in reducing sensory neuropeptide release has been offered in cluster headache patients, where its administration acutely reverted the increase in CGRP-LI observed during nitrate-induced attacks [17].

The ability of sumatriptan and dihydroergotamine to inhibit CGRP-LI release from slices of rat trigeminal ganglia after EFS and high K^+ concentrations is consistent with similar data obtained in rats in vivo [13] or in trigeminal primary sensory neurons grown in culture [28]. The present results indicate that $5\text{-}HT_{1B/D}$ receptors are expressed in trigeminal ganglion cell bodies with proximal nerve trunks attached. More importantly, the results indicate that at this location these receptors are functional, as they are in the

very terminal region of the nerve fiber. This observation strengthens the proposal of the present model as a suitable one for the study of the pharmacological modulation of the release of sensory neuropeptides. Thus, the present model may be used for the scrutiny of the pharmacodynamic properties of new drugs that may act via 5-$HT_{1B/D}$ receptors. It may also be used to examine the ability of additional receptors to exert an inhibitory role on the release of the potent pro-algesic and pro-inflammatory sensory neuropeptides.

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COMMENT

Human disease models often attempt to reproduce pathophysiological conditions in animals to measure the same biological parameters observed in clinical situations. However, the diagnosis of migraine headache relies heavily on the history of symptoms and reporting of a subjective experience which obviously cannot be reproduced in animals. None of the experimental models proposed to date are unequivocally considered to be entirely fitting the pathophysiology of migraine.

Although the issue of neurogenic [1] versus vascular [2] mechanisms is still unresolved, there is a consensus regarding the activation of trigeminal structures as essential for the genesis of the attack [3]. According to this view, the vascular component seems to represent a target of the mediators released by trigeminal nerve endings. Experimental models (based on the involvement of the trigeminovascular system in migraine) have evaluated plasma protein extravasation [4], or cranial vasodilatation [5] induced by trigeminal stimulation. In both models, 5-$HT_{1B/D/F}$ agonists endowed with antimigraine activity have exerted inhibitory effects on the consequences of trigeminal stimulation. Nevertheless, up to now, the pharmacological screening of antimigraine 5-HT agonists has been mostly based on their direct vasoconstrictor properties (mediated by 5-HT_{1B} receptors). It should be considered that, if their vasoconstrictor efficacy represents a good predictor of the antimigraine effect, it is also potentially harmful at the coronary level. Also, stimulation of presynaptic 5-HT_{1D} and/or 5-HT_{1F} receptors inhibits the release of neuropeptides (substance P, CGRP, neurokinin A) from trigeminal axons and blocks neurotransmission. Therefore, investigating only the direct vascular effects of a given compound provides less than complete information. In this context, determination of the amount of neuropeptide released from trigeminal perivascular nerves would be of interest, but is not always feasible.

In this issue, Tognetto et al. [6] report on the release of neuropeptides from slices of rat trigeminal ganglia. According to their data, trigeminal primary sensory neurons, known to release neuropeptides from peripheral and central endings, released neuropeptides also from the cell bodies and/or the proximal axons. In fact, they show that CGRP and SP immunoreactivity can be released from trigeminal slices after electrical stimulation, KCl-induced depolarization or capsaicin treatment. They validate the model by showing that the increase in neuropeptide immunoreactivity is reversible upon removal of the stimulus and that the phenomenon is not solely related to the rat species, since electrical stimulation evokes a robust increase in CGRP also in slices from guinea pig trigeminal ganglion. Furthermore, the release of neuropeptide immunoreactivity implies a neurosecretory mechanism, since it is inhibited by Ca^{2+} removal or blockade of Na^{+} or Ca^{2+} channels. Interestingly, both dihydroergotamine and sumatriptan are able to inhibit the release of neuropeptide immunoreactivity, presumably through stimulation of prejunctional 5-$HT_{1B/D}$ receptors. Future studies should attempt to extend this observation to other serotoninergic drugs, and, eventually, to other neuropeptides, such as neurokinin A. If the phenomenon is proven to occur through stimulation of prejunctional receptors, this model could provide a tool for studying new antimigraine compounds specific to 5-HT and other prejunctional receptors.

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