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Peripheral and central alterations of pituitary adenylate cyclase activating polypeptide-like immunoreactivity in the rat in response to activation of the trigeminovascular system

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ABSTRACT

Pituitary adenylate cyclase activating polypeptide (PACAP) is present in the cranial arteries and trigeminal sensory neurons. We therefore examined the alterations in PACAP-like immunoreactivity (PACAP-LI) in a time-dependent manner in two rat models of trigeminovascular system (TS) activation. In one group chemical stimulation (CS) was performed with i.p. nitroglycerol (NTG), and in the other one the trigeminal ganglia (TRG) were subjected to electrical stimulation (ES). The two biologically active forms, PACAP-38 and PACAP-27, were determined by means of radioimmunoassay (RIA) and mass spectrometry (MS) in the plasma, the cerebrospinal fluid (CSF), the trigeminal nucleus caudalis (TNC), the spinal cord (SC) and the TRG. The tissue concentrations of PACAP-27 were 10 times lower than those of PACAP-38 in the TNC and SC, but about half in the TRG. PACAP-38, but not PACAP-27, was present in the plasma. Neither form could be identified in the CSF. PACAP-38-LI in the plasma, SC and TRG remained unchanged after CS, but it was increased significantly in the TNC 90 and 180 min after NTG injection. In response to ES of the TRG, the level of PACAP-38 in the plasma and the TNC was significantly elevated 90 and 180 min later, but not in the SC or the TRG. The alterations in the levels of PACAP-27 in the tissue homogenates in response to both forms of stimulation were identical to those of PACAP-38. The selective increases in both forms of PACAP in the TNC suggest its important role in the central sensitization involved in migraine-like headache.

1. Introduction

The sensory afferent innervation of the cerebral vessels is provided only by the trigeminovascular system (TS), which plays a pivotal role in the physiological regulation of cerebrovascular structures [47]. The TS is composed of pseudounipolar neurons whose cell bodies are located in the trigeminal ganglion (TRG) [47]. These neurons constitute the major afferent pain pathway between the cranial vessels and the nuclei in the brainstem and in the upper regions of the spinal cord. The peripheral branches reach the cranial vessels and meningeal tissues. The central terminals of these fibers project to the nociceptive second-order neurons in the trigeminal nucleus caudalis (TNC) located in the brainstem and more caudally in the upper regions of the spinal cord. The sensory trigeminal unit is probably controlled by the descending pathways from the dorsal nucleus raphe, the periaqueductal gray matter and the locus coeruleus [82].

The pituitary adenylate cyclase activating polypeptide (PACAP) is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family that is widely distributed in the human organisms [2,91]. PACAP was discovered on the basis of its ability to increase adenylate cyclase activity in rat pituitary cells, and was first isolated from the ovine hypothalamus in 1989 [49]. The gene of PACAP is localized on the short arm of chromosome

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18 [29] and the peptide occurs in two biologically active amidated forms, containing 38 and 27 amino acids: PACAP-38 and PACAP-27. PACAP-38 is the predominant form, accounting for 90% of the total PACAP content in most mammalian tissues, but it is rapidly metabolized and its plasma elimination half-life is less than 5 min [8]. PACAP is widely expressed in the central nervous system [46,88], in peripheral organs [69], in endocrine glands [14,36], and in secretions from the exocrine glands [7], thereby functioning as a pleiotropic peptide [50,79]. It is a hypophysiotropic hormone [35], a neurotransmitter and a neuromodulator in the nervous system [22], and it exerts neuroprotective [70], antiapoptotic [78] and differentiation-inducing effects in the developing nervous system [57,95]. Furthermore, it serves important regulatory and protective roles in the gastrointestinal [16], cardiovascular [25,66,94], reproductive [4,38] and respiratory systems [13]. The effects of PACAP are mediated through three receptors: $VPAC_1$ (previously designated the VIP, VIP1 or PACAP type II receptor), $VPAC_2$ (known as the VIP2 or PACAP type III receptor) and PAC_1 (formerly known as PACAP type I receptor); the latter has 1000-fold higher specific affinity for both forms of PACAP than for VIP [37,77]. The binding of PACAP to its receptors induces two main signal transduction pathways: (1) through Gs-protein activation, it stimulates the activity of adenylate cyclase, leading to increased cyclic adenosine monophosphate (cAMP) levels; (2) via the Gq-protein-coupled process, it activates phospholipase C and increases the intracellular calcium (Ca^{2+}) concentration. The increased cAMP level can activate a number of kinases, which exert a variety of physiological and pathophysiological effects by phosphorylating certain proteins [77,91].

The role of PACAP in vasodilatation [11,19] and nociceptive processes [23,53,54,74,75,98,99] has been confirmed in several studies. The presence of this peptide has been demonstrated in the trigeminal system [3,55,83,88]. The co-localization of nociceptin and PACAP has been described, but their relationship is not known. An investigation of human TRGs revealed that ~68% of the nociceptin-positive cells contained PACAP [24]. In another study, moderately dense calcitonin gene-related peptide (CGRP) and PACAP-containing fibers were observed adjacent to numerous substance P (SP)-immunoreactive (-ir) fibers, but VIP-ir fibers were not seen in the TNC or at the cervical$_1$–cervical$_2$ (C_1–C_2) levels of the spinal cord [67,88]. The coexistence of PACAP and SP has also been reported [48,81]. Moreover, PACAP coexists with CGRP in sensory ganglia and nerve plexuses of inner organs [21]. A clinical study has revealed that intravenously administered PACAP-38 induces migraine-like attacks in healthy volunteers and patients with migraine without aura [76], similarly to the effect of nitroglycerol (NTG) in causing headache [59,80]. The receptors of PACAP have therefore been implicated in migraine pathophysiology, as potential therapeutic targets, but there are no direct experimental data to confirm this theory [77]. It is assumed that PACAP may be one of the mediators involved in the mechanisms of TS activation and the modulation of extracranial or dural trigeminal nociceptors.

A commonly applied and well-established animal model of TS activation is the systemic administration of NTG. Extensive literature is available on this field regarding the mechanisms, the good reproducibility and the human relevance [6,10,39,58,87]. The effect of NTG is based on the release of nitrogen oxide (NO), which causes rapid vasodilatation. NO is an endogenous transmitter, formed during the conversion of L-arginine to citrullin on the action of nitric oxide synthase (NOS). The NOS molecule is one of the markers of trigeminal activation, since NO itself is a very unstable gaseous substance that is difficult to detect. The neuronal isoform (nNOS) is the most important enzyme from the aspect of sensory information in trigeminal pain processing, because it is abundant in the superficial layers of the dorsal horn of the spinal cord [61,63,85].

Another possibility via which to develop an activated state of the TS is electrical stimulation (ES) of the TRG. This is a well-described, widely used and generally certified method of TS activation with a broad range of stimulation parameters [1,9,41,45,72,73,84]. A similar effect can be evoked by ES of the superior sagittal sinus. Besides direct stimulation of the peripheral trigeminal afferents, ES can cause mast cell degranulation in the dura mater and the tongue. Pronounced neurogenic inflammation (vasodilatation and plasma protein extravasation) therefore develops on the brain surface. These responses are explained by the release of inflammatory mediators, e.g. various vasoactive neuropeptides. These phenomena can trigger general neuronal activity in the area of the trigeminal complex or changes in blood flow [68,96] and even induce structural alterations in the nerve terminals [9,17,32,33].

The present goal was to investigate PACAP-38- and PACAP-27-like immunoreactivities (PACAP-38-LI and PACAP-27-LI) in the peripheral and central regions of the TS in a time-dependent manner following two types of TS activation in the rat. In order to understand its role in the activation of the TS, PACAP-38/27 levels were measured in venous blood plasma (the cranial vena cava), the area of second-order sensory neurons (TNC), the spinal cord (C_3–C_4), the TRG and the cerebrospinal fluid (CSF) (the suboccipital cistern).

2. Materials and methods

2.1. Animals

Fifty-nine young adult Sprague-Dawley rats of either sex (8–12 weeks old, 250–350 g body weight) were used in these studies: 28 in the NTG-induced TS activation model, 20 in the electrical TRG-stimulation model, and 11 as intact animals in the control group. The animals were bred and maintained under laboratory conditions on a 12-h dark 12-h light cycle at 24–25 °C and ~80% relative humidity in the Laboratory Animal House of the Department of Neurology in Szeged. Standard rat chow and tap water were available ad libitum.

2.2. Ethics

All experimental procedures performed in this study complied fully with the guidelines of Act 1998/XXVIII of the Hungarian Parliament on Animal Protection and the Decree on Scientific Procedures in Animal Experiments (243/1988), and with the recommendations of the International Association for the Study of Pain [102] and the European Communities Council (86/609/ECC). The studies were in harmony with the Ethical Codex of Animal Experiments and were approved by the Ethics Committee of the Faculty of Medicine, University of Szeged.

2.3. CS of the TS

Three groups were involved in the NTG-induced CS studies. One group of 11 animals remained intact. In two other groups, 14 animals per group received a single i.p. injection of NTG (prepared from Nitrolingual Pumpspray, Pohl-Boskamp GmbH, Germany) in a dose of 10 mg/kg (0.13 ml/100 g of a 7.68 mg/ml solution) to induce CS of the TS. In rats, NTG in the dose mentioned above, as a massive stimulus for the TS, can trigger physiological (arterial diameter, pulsation and blood flow [26]) and molecular (c-fos, CGRP, SP, nNOS and CaMKII [5,18,58,62,86,101]) responses that resemble a common manifestation of activated TS. Before blood sampling, the animals were anesthetized with i.p. 4% chloral hydrate solution (in a dose of 10 ml/kg), which provided stable, deep anesthesia. Blood sampling followed immediately in the intact group, but only 90 min

or 180 min after NTG administration in the two other groups. In preliminary experiments, sampling was also carried out after 15 and 30 min (data are shown in the case of plasma), but in view of the absence of changes in PACAP-38-LI at these times, this was not done later. Blood samples (5 ml per animal) were taken from the right cranial vena cava into ice-cold glass tubes containing ethylenediaminetetraacetic acid (12 mg) and the protease inhibitor aprotinin (Trasylol, 1200 IU). Samples were kept at 4 °C until the blood plasma was separated by centrifugation (5000 rpm for 10 min at 4 °C). Before cupping, CSF (~150 μl per animal) was taken from the suboccipital cistern, and following cupping different nerve structures (the TNC, spinal cord and TRG) were excised from the animals at the 90 or 180 min time points. Samples were stored at −80 °C until the measurement of PACAP-38-LI and PACAP-27-LI by radioimmunoassay (RIA) and determination of these peptides by mass spectrometry (MS).

2.4. ES of the TS

Five animal groups were created for these examinations: 11 rats served as non-stimulated intact animals; two groups of 5 rats each were followed up after sham stimulation until 90 and 180 min, respectively; and two groups of 5 rats each were investigated at 90 and 180 min after ES of the TRG. In earlier experiments, sampling was carried out after 30 min too (data are shown in the case of plasma), but as there was no change in PACAP-38-LI at this time, this was not done later. First, the rats were deeply anesthetized with i.p. 4% chloral hydrate solution (in a dose of 10 ml/kg) and the anesthesia was maintained throughout the experiment. The animals were placed in a stereotaxic setup and the head was fixed. After removal of the scalp, the localization of the TRG from the bregma was measured with micromanipulators according to the Watson-Paxinos Rat Brain Atlas (anteroposterior: 3.2 mm; mediolateral: 2.9 mm). Following drilling of the skull at the assigned point, the stimulating macroelectrode was passed into the brain to reach the TRG. The TRG was stimulated according to the following parameters: 30 min duration; train rate: 2 s × 1; train duration: 5 ms × 1; stimulation rate: 10 pps × 1; delay: 5 ms × 1; duration: 5 ms × 1; current (mA%): 20 × 5 mA = 1 mA%; stimulation mode: TWIN-PULSES. This ES method (30 min, 10 Hz, 5 ms duration, 1 mA) can induce massive neuropeptide release from the pseudounipolar TRG neurons [30,31,34]. This neuropeptide depletion can be attributed to the more rapid firing of cells caused by the relatively high current and frequency and the long duration of the stimulation [71]. In cases of sham stimulation, the electrode was positioned in the same way at the same location, but no current was applied. CSF, blood samples and neural tissues were taken, stored and analyzed as described above.

2.5. RIA determination of plasma, CSF and tissue PACAP-38-LI and PACAP-27-LI

Plasma and CSF concentrations of PACAP-38 and PACAP-27 were determined with specific and sensitive RIA techniques developed in our laboratory [27]. The "88111-3" PACAP-38 and the "88123-3" PACAP-27 antisera were raised in rabbits with synthetic peptides conjugated to bovine serum albumin (BSA) or thyroglobulin with glutaraldehyde or carbodiimide. The high specificity and C-terminal sensitivity of this antibody were confirmed by cross-reactivity studies: no cross-reactivity was found with PACAP-27 in the PACAP-38 assay, with PACAP-38 in the PACAP-27 assay, or with other neuropeptides in either case. Following centrifugation of the blood samples (2000 rpm at 4 °C for 10 min), the peptide was extracted from the plasma into 3 volumes of absolute alcohol. After precipitation and a second centrifugation (2000 rpm at 4 °C for 10 min), the samples were dried under a nitrogen flow and resuspended in 300 μl assay buffer before RIA determination so as to achieve a 10 times higher concentration for the RIA procedure [27,54]. Brain segments (TRG, TNC and C_3–C_4 spinal cord segments) were frozen and stored at −80 °C until further processing. The samples were weighed and homogenized in 1 ml ice-cold bidistilled water with a manual potter homogenizer. The homogenates were centrifuged at 10,000 rpm for 10 min and then at 12,000 rpm for another 10 min, and 70 μl samples of the supernatants were used for RIA measurements.

The tracers were mono-^{125}I-labeled peptides prepared in our laboratory. Synthetic peptides were used as RIA standards in concentrations ranging from 0 to 1000 fmol/ml. The assay was prepared in 1 ml 0.05 M (pH 7.4) phosphate buffer containing 0.1 M sodium chloride, 0.25% (w/v) BSA and 0.05% (w/v) sodium azide. The antiserum (100 μl, 1:10,000 dilution), the RIA tracer (100 μl, 5000 cpm/tube) and the standard or unknown samples (100 μl) were measured into polypropylene tubes with the assay buffer. After incubation for 48–72 h at 4 °C, the antibody-bound peptide was separated from the free peptide by addition of 100 μl separating solution (10 g charcoal, 1 g dextran and 0.5 g commercial fat-free milk powder in 100 ml distilled water). Following centrifugation (3000 rpm at 4 °C for 15 min), the contents of the tubes were gently decanted and the radioactivity of the precipitates was measured in a gamma counter (Gamma, type: NZ310). The PACAP-38 and PACAP-27 concentrations of the unknown samples were read from calibration curves.

2.6. Examination of PACAP-38 and PACAP-27 in the rat plasma and CSF by MS

Identification of PACAP-38 and PACAP-27 in the rat plasma and CSF samples in comparison with standard solutions was performed with matrix-assisted laser desorption ionization time of flight (MALDI TOF) MS. The quasimolecular ions of the PACAP-38 Na^+ adduct (MW: 4558.7) and PACAP-27 (MW: 3147.6) or its $[M+Na]^+$ were determined. The aqueous solutions of the PACAP-38 and the PACAP-27 standards and the examined samples were loaded onto the target plate (MTP 384 massive target T, Bruker Daltonics, Bremen, Germany) by mixing 1 μl of each solution with the same volume of a saturated matrix solution, prepared freshly every day by dissolving α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile/0.1% TFA (1/2, v/v) [23].

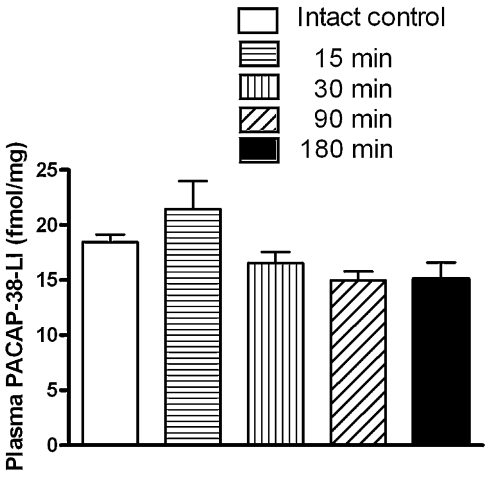

Fig. 1. PACAP-38-like immunoreactivity (PACAP-38-LI) determined by RIA in rat plasma 15, 30, 90 and 180 min after i.p. injection of 10 mg/kg nitroglycerol. Plasma samples of untreated intact rats served as control. Each column denotes the mean + SEM of the results on $n = 11$–28 animals. Significant differences were not observed with one-way ANOVA followed by Tukey's post hoc test.

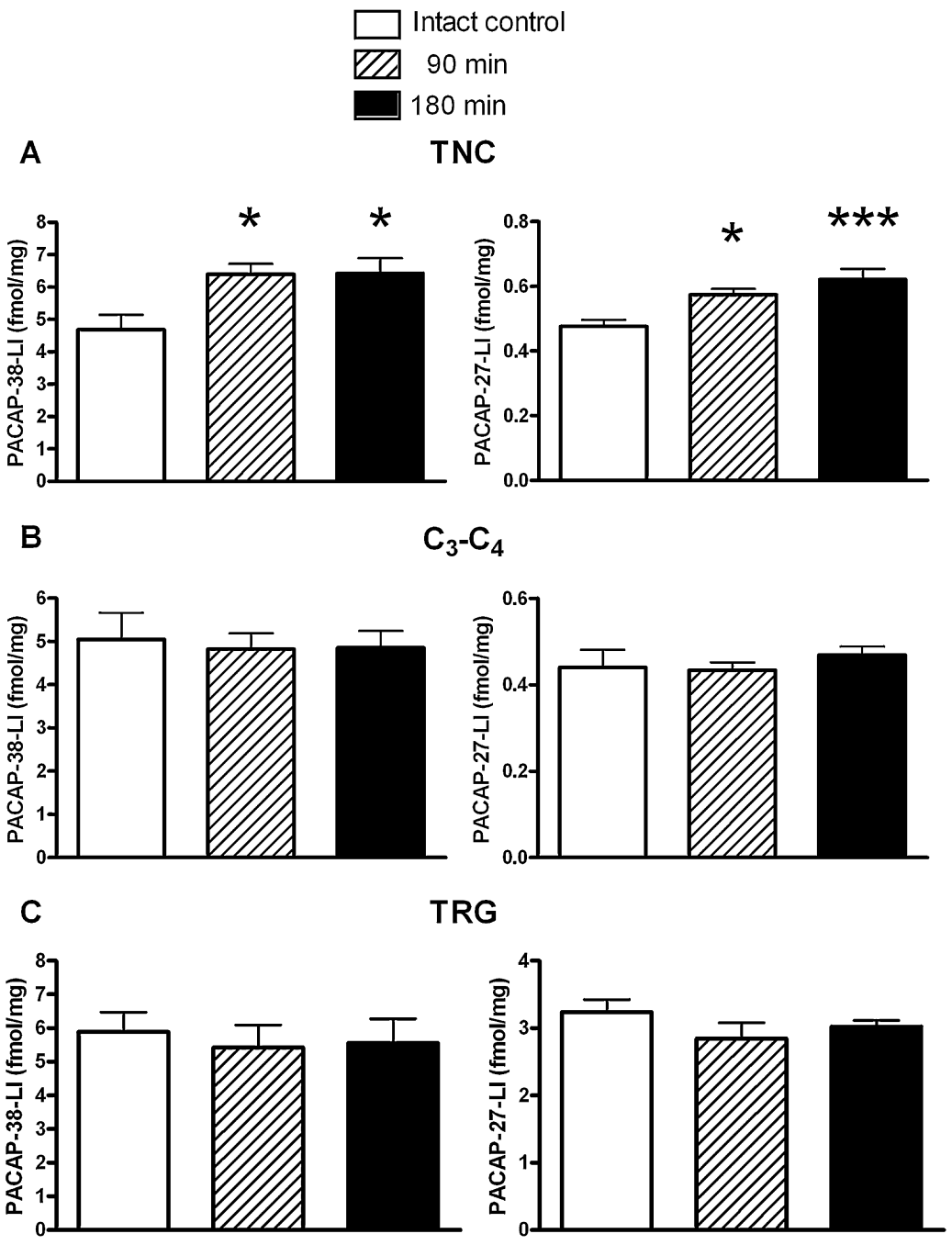

Fig. 2. PACAP-38-like and PACAP-27-like immunoreactivities (PACAP-38-LI and PACAP-27-LI) determined by RIA in homogenates of (A) the trigeminal nucleus caudalis (TNC), (B) the C_3–C_4 spinal cord segments, and (C) the trigeminal ganglia (TRG) of the rat 90 and 180 min after 10 mg/kg i.p. nitroglycerol injection. The respective brain segments of untreated intact rats served as control. Each column denotes the mean + SEM of the results on $n = 11$–28 animals; $^{*}p < 0.05$, $^{***}p < 0.001$ vs. intact control (one-way ANOVA followed by Tukey's post hoc test).

The CSF samples were desalted and cleaned with 0.1% TFA solution with the use of $ZipTip_{18}$ pipette tips (Millipore Kft., Hungary). The purified proteins and peptides were eluted directly onto the MALDI target plate with 3 μl of acetonitrile/0.1% TFA (50/50, v/v) solution by mixing 1 μl of the saturated matrix solution described above.

The ions were accelerated under delayed extraction conditions (200 ns) in positive ion mode with an acceleration voltage of 20.00 kV; each spectrum was detected in linear mode. The instrument uses a 337 nm pulsed nitrogen laser, model MNL-205MC (LTB Lasertechnik Berlin GmbH., Berlin, Germany). External calibration was performed in each case with #206195 Peptide Calibration Standards (Bruker Daltonics, Bremen, Germany). Protein masses were acquired in the range of 1000–8000 *m*/*z*. Each spectrum was produced by accumulating data from 300 consecutive laser shots. The Bruker FlexControl 2.4 software was used for control of the instrument and the Bruker FlexAnalysis 2.4 software for spectrum evaluation.

2.7. Statistical analysis

Data are presented as means + SEM of the results on $n = 11$–28 animals. Statistical analysis was performed with one-way analysis

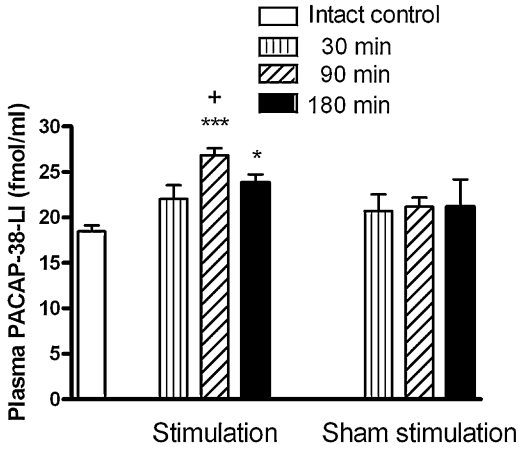

Fig. 3. PACAP-38-like immunoreactivity (PACAP-38-LI) determined by RIA in the rat plasma 30, 90 and 180 min after electrical stimulation of the TRG (10 Hz, 1 mA, 30 min). The plasma samples of untreated intact rats and sham-stimulated rats served as controls. Each column denotes the mean + SEM of the results on $n = 11–20$; $^{*}p < 0.05$, $^{***}p < 0.001$ vs. intact control; $^{+}p < 0.05$ vs. sham-stimulated control at the respective time (one-way ANOVA followed by Tukey's post hoc test).

of variance (ANOVA) followed by Tukey's post hoc test. Levels of probability $p < 0.05$ were considered significant.

3. Results

3.1. Changes in PACAP-38/27-LI in rat plasma and different brain regions in response to CS of the TS

The level of PACAP-38-LI in the systemic circulation of intact, untreated rats, 18.5 ± 3.6 fmol/mg, was not significantly changed within the 180-min examination period by CS of the TS with 10 mg/kg i.p. NTG (Fig. 1). PACAP-27-LI was not measurable in the rat plasma; it was below the detection limit of the assay even when the total peptide content was extracted from a volume of 4 ml.

Both PACAP-38-LI and PACAP-27-LI could be reliably measured by RIA in the homogenates of different rat brain regions related to the trigeminal system. Their concentrations were ~5–6 fmol/mg and ~0.4–0.6 fmol/mg wet tissue respectively, in each of the TNC, the C_3–C_4 spinal cord segments and the TRG. NTG injection evoked significant increases in PACAP-38-LI at both 90 min and 180 min in the TNC, but not in the other two examined areas. The concentrations of PACAP-27-LI were about 10 times lower than those of PACAP-38-LI in both the TNC and the C_3–C_4 spinal cord regions. The level of the shorter form was approximately half that of the longer one in the TRG. The NTG-induced alterations in PACAP-27-LI in each region were identical to the changes in PACAP-38-LI (Fig. 2).

3.2. Changes in PACAP-38/27-LI in rat plasma and different brain regions in response to ES of the TS

In contrast with what was observed on CS of the TS with NTG, ES of the right TRG led to a significant, ~30% elevation of the plasma PACAP-38-LI 90 min later. This elevation subsequently declined somewhat, but still remained significant at 180 min. Sham stimulation (electrode insertion without ES) did not influence the PACAP-38-LI in the systemic circulation (Fig. 3).

Similarly to the effect of the NTG injection, electrical TRG stimulation gave rise to significant increases in both PACAP-38-LI and PACAP-27-LI in the TNC after 180 min, whereas no change was observed in the C_3–C_4 and the TRG regions. No change was detected in the sham-stimulated group (Fig. 4).

3.3. Identification of PACAP-38 and PACAP-27 in rat plasma and CSF

PACAP-38 was clearly identified by MS at m/z 4535.3 (PACAP-38 H^+ adduct) in the intact rat plasma samples, but PACAP-27 was not detectable (Fig. 5C) relative to the standard spectra (Fig. 5A and B). However, neither form could be found in the CSF samples obtained from any group (Fig. 5D). RIA measurements confirmed the lack of PACAP-38-LI and PACAP-27-LI in the CSF (data not shown).

4. Discussion

Numerous animal experiments [23,53,74,99] and some clinical studies [76,77] have pointed to the key role of PACAP in nociceptive signaling mechanisms. Although its involvement in migraine has recently been indicated by human data [76], and its immunohistochemical localization has been described in the TS [88], the present paper provides the first experimental evidence that its concentration is specifically altered in the TNC and the plasma in response to the activation of the TS in rat models. Our results support the neuropeptide theory of the development of activation of the TS. Both peripheral and central sensitization occurred in our models, suggesting the complexity of the activated TS.

The divergent results observed in the different brain regions can be explained by the differences in the activation mechanisms of the TS in the two models.

The only known effect of NTG is the release of NO, which is responsible for the vasodilating action in the immediate dull headache after NTG administration. The delay in the action of NO triggers a typical attack in migraineurs, which is possibly related to the activation of trigeminal Aδ and C fibers leading to central sensitization at the level of the TNC in the TS [61]. In our experiment, this CS was not strong enough to generate pronounced alterations in plasma PACAP-38-LI. The higher PACAP-38-LI 15 min after NTG injection as compared with the control groups is explained by the acute and short-term effect of NO. In another study, we showed that the systemic blood pressure decrease occurs in the first 10 min following NTG injection, after which it remains unchanged [44]. These phenomena are manifested in alterations in vessel wall tension, which can be one of the triggers of the pseudounipolar neurons of the TRGs, since the intracranial vasculature is mainly innervated by the trigeminal nerves [47]. The initial vessel wall tension changes, as a trigger, stimulate the peripheral trigeminal nerve terminals, and therefore a small amount of PACAP-38 can be released into the systemic circulation. Later, when the balance starts to evolve again from the aspect of the blood pressure, the vessel wall tension will not change as much as earlier. It does not function as a trigger, so it cannot evoke PACAP-38 release. The other explanation of the decreasing PACAP-38-LI 30, 90 and 180 min after NTG injection is the short (less than 5-min) plasma elimination half-life of PACAP-38 in the plasma.

Central sensitization is generally confirmed in the NTG model, as observed in our experiment by the elevated PACAP-38-LI and PACAP-27-LI in the TNC, but not in the plasma. NTG evokes migraine attacks [15,80] and develops sensitization [43] in human studies. Similarly, in animal experiments, NTG activates second-order neurons and selectively elevates the levels of nNOS [61] and Ca^{2+}/calmodulin-dependent protein kinase II [60] immunoreactivity, which are involved in nociceptive central sensitization. The NTG-induced PACAP increase is likely to have an important role in the trigeminal activation and sensitization processes in the region of the TNC. The fact that systemic administration of PACAP can evoke sensitization and migraine-like attacks in migraineurs [76] is in line with the results of our animal experiments. Moreover, PACAP release in the dorsal horn has been reported in other

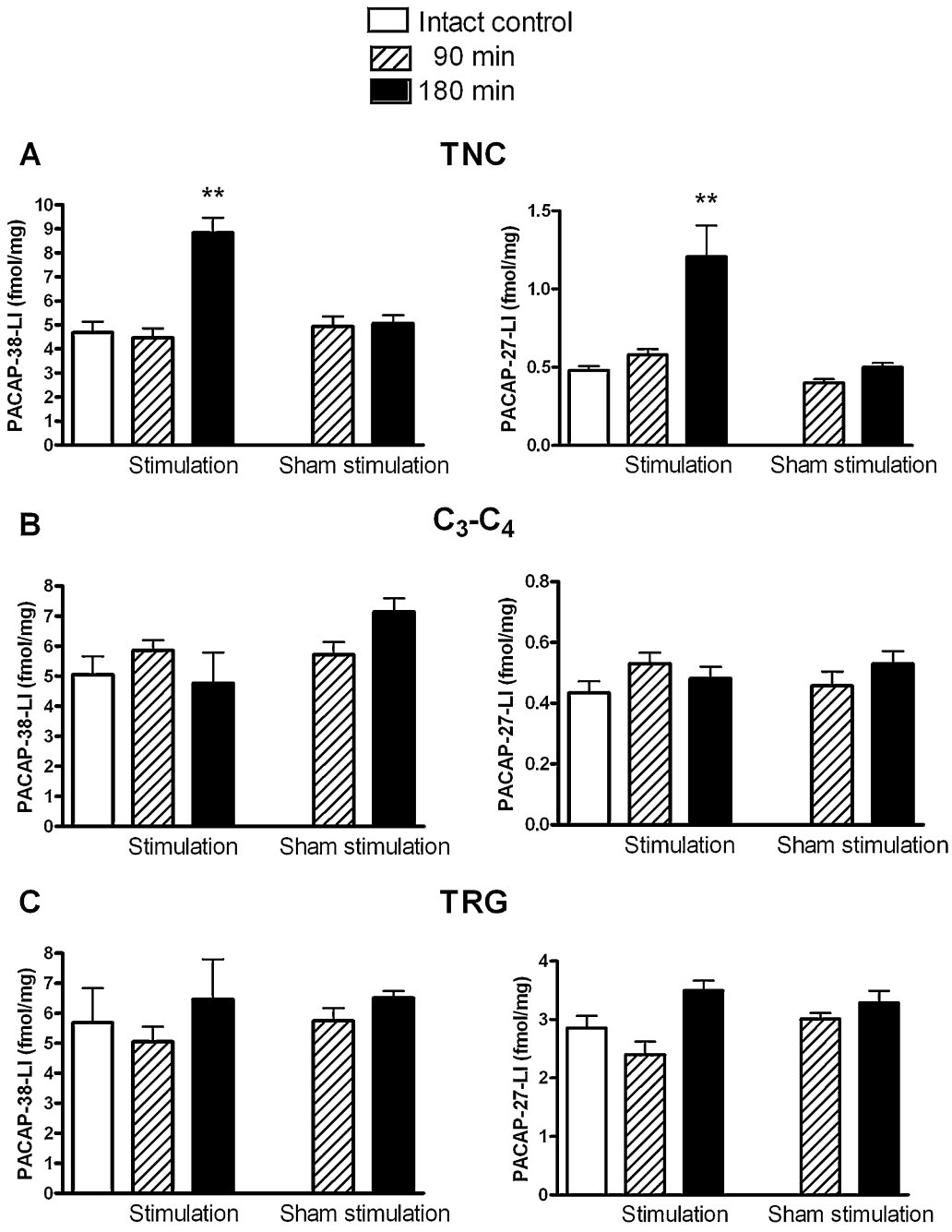

Fig. 4. PACAP-38-like and PACAP-27-like immunoreactivities (PACAP-38-LI and PACAP-27-LI) determined by RIA in homogenates of (A) the trigeminal nucleus caudalis (TNC), (B) the C_3–C_4 spinal cord segments, and (C) the trigeminal ganglia (TRG) of the rat 90 and 180 min after electrical stimulation of the TRG (10 Hz, 1 mA, 30 min). The respective brain segments of untreated intact rats and sham-stimulated rats served as controls. Each column denotes the mean + SEM of the results on $n = 11$–20 animals; $^{**}p < 0.01$ vs. intact control (one-way ANOVA followed by Tukey's post hoc test).

experimental conditions of peripheral stimulation [12,20,56,65,103]. The potential relationship between PACAP and pain is enhanced by the co-localization and functional link of the NOS and PACAP systems, which has been described in a variety of studies [40,42,52,75,89,90].

The ES of the TRG caused significant elevations of PACAP-38-LI in the blood plasma and the TNC, which may be a result of PACAP release from both the peripheral [24,51] and presumably the central terminals of the primary sensory neurons. This is in line with previous findings that capsaicin can induce PACAP release from peripheral sensory nerves [100]. The fact that the highest PACAP level in the circulation was measured 90 min after the ES is probably due to its release from the peripheral terminals of the activated neurons peaking at this time. The PACAP elevation was still significant at 180 min, but was then tending to decrease due to peptide depletion.

The PACAP-38-LI increase in the plasma preceded the changes seen in the TNC after ES, which suggests a rapid peripheral release, followed by activation and sensitization of the second-order trigeminal neurons. The ES-induced activation of the TS is followed by enhanced PACAP release from the central terminals of the TRG neurons in the TNC. A similar change was reported in the cyclophosphamide-induced chronic cystitis model, where the PACAP-LI increased significantly in the spinal segments and dorsal root ganglia (DRG) involved in micturition reflexes [93]. This PACAP release is also in accordance with the PACAP-LI elevation noted

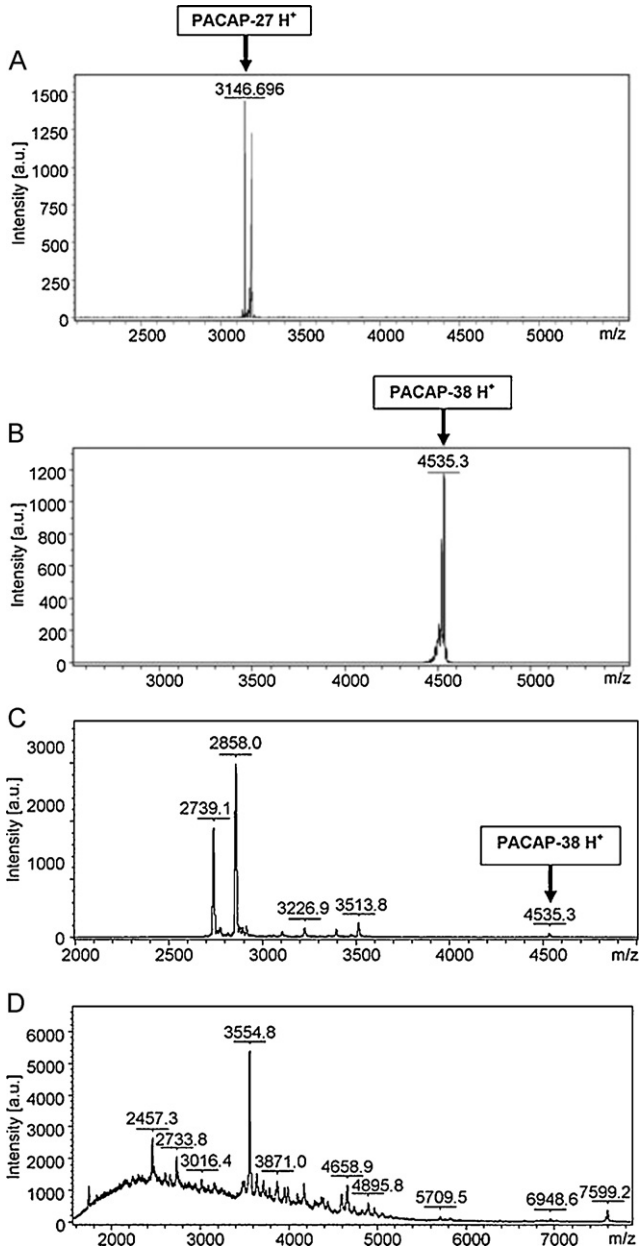

Fig. 5. Identification of PACAP-38 in (B) the standard and (C) intact rat plasma samples by MS at m/z 4535.3 Da, representing the average mass of the protonated quasimolecular ion of PACAP-38. Neither the other biologically active form, PACAP-27 (3147.6 Da), nor its $[M+Na]^+$ could be detected as compared to the (A) PACAP-27 standard. Panel D is a MALDI TOF spectrum of the intact rat CSF sample in positive ion mode using linear detection, where the characteristic peaks of PACAP-38 (4535 Da) and/or PACAP-27 (3147.6 Da) were not observed.

after capsaicin administration into the subarachnoid space [100]. Briefly, the up-regulation of this peptide may indicate a general trigeminal activation.

The most noteworthy result was the elevation of both PACAP-38-LI and PACAP-27-LI in the TNC, which occurred selectively after both CS and ES of the TS. We have recently reported that i.p. injected PACAP evokes a marked photophobia, meningeal vasodilatation and an increased number of c-fos-positive activated neurons in the TNC of wild-type, but not PACAP-deficient mice [44]. These data are in complete agreement with our present conclusion that PACAP released in the TNC is responsible for central sensitization of the TS. Molecular changes restricted to this brain area were observed in our previous experiments: for c-fos and NOS-immunoreactivity in the NTG model [61], and for CGRP on ES in the TRG model [33].

Our experiments did not reveal PACAP changes of any kind in the TRG in either model. Increased levels of PACAP expression in the sensory neurons of the rat DRG have been described several days after irreversible peripheral nerve damage (transsection) [99] and nerve compression [64]. The lack of change in PACAP expression in the TRG in our experiments might be related to the fact that we applied acute stimulations, and not peripheral nerve damage, or that the short stimulation period and latency was not sufficient to cause substantial expression changes.

An earlier study revealed decreased somatostatin- and beta-endorphin-like immunoreactivities of plasma or CSF obtained by suboccipital puncture, while the neuropeptide Y-like immunoreactivity did not change during the attack period in patients suffering from common migraine [92]. The present MS results confirmed the RIA detection of the presence of PACAP-38 in the rat plasma. In contrast, the presence of PACAP in the CSF was not detected with either the highly sensitive and specific RIA technique or MS, which suggests that PACAP is unable to cross the intact blood-CSF barrier. However, one paper has reported the presence of PACAP in an artificial CSF perfusate in a specific rat experimental setup, where capsaicin was added to the perfusate. Nevertheless, under these conditions, disruption of the blood-CSF barrier cannot be excluded [100]. PACAP-27 was not detectable in either the plasma or the CSF, which might be explained by the generally significantly lower concentration (10–100 times lower) of PACAP-27 in the mammalian tissues examined so far [91].

The altered PACAP levels in our activated TS models can be compared with the role of this peptide in previously studied pain mechanisms. A number of investigations have demonstrated various actions of PACAP in nociceptive signaling processes. Intrathecally administered PACAP dose-dependently decreased flinching of the hindpaw in the formalin test [97,99]. In contrast, PACAP induced hyperalgesia after administration into the mouse spinal cord [53]. There is evidence that PACAP and its receptors can modulate the activity of single multireceptive dorsal horn neurons in response to sensory inputs [12]. The up-regulated expression of the PAC_1 receptor in DRGs in the $lumbar_5$ segment of the spinal cord was observed following unilateral adjuvant/induced inflammation, while the level of the $VPAC_1$ receptor agonist was diminished, whereas that of the $VPAC_2$ receptor agonist was increased in a neuropathic pain state caused by direct trauma or a compression nerve injury [12]. The neuroregulatory functions of the PAC_1 receptor were demonstrated in a study where the chronic nociceptive response of PAC_1 receptor knock-out mice was markedly reduced in formalin, thermal laser and mechanical stimulation-initiated models of inflammation [28]. The inflammatory/neuropathic pain due to the effect of carrageenan and spinal nerve transection can be suppressed by the absence of the PACAP gene. Intrathecal administration of N-methyl-D-aspartic acid (NMDA) did not cause mechanical allodynia in PACAP knock-out mice, but it was evoked by the application of PACAP with NMDA, which suggests that this peptide can be the integrator between nociception and central sensitizational processes [42]. Nevertheless, the typical sensitization phenomena of migraine can be evoked by the systemic application of PACAP-38 in humans [76].

5. Conclusions

Both PACAP-38-LI and PACAP-27-LI are specifically elevated in the TNC in response to CS and ES of the TS. Furthermore, a marked elevation of only PACAP-38 was detected in the systemic circulation and only after electrical TRG activation. The results indicate that this peptide is closely connected with the NO system, and PACAP might

therefore play a pivotal role in nociception in the TS. These data facilitate the understanding of the mechanisms of trigeminal activation, and answer certain questions relating to clinically relevant sensitization processes. They also provide perspectives concerning the identification of new targets for the treatment of migraine.

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Glossary

ANOVA: analysis of variance
C_1–C_2: cervical$_1$–cervical$_2$ segments of spinal cord
C_3–C_4: cervical$_3$–cervical$_4$ segments of spinal cord
cAMP: cyclic adenosine monophosphate
CGRP: calcitonin gene-related peptide
CSF: cerebrospinal fluid
DRG: dorsal root ganglion
ES: electrical stimulation
MALDI TOF: matrix-assisted laser desorption ionization time-of-flight
MS: mass spectrometry
NMDA: N-methyl-D-aspartic acid
nNOS: neuronal nitric oxide synthase
NO: nitrogen oxide
NOS: nitric oxide synthase
NTG: nitroglycerol
PAC_1: PACAP type I receptor
PACAP: pituitary adenylate cyclase activating polypeptide
PACAP-27: pituitary adenylate cyclase activating polypeptide-27
PACAP-27-LI: pituitary adenylate cyclase activating polypeptide-27-like immunoreactivity
PACAP-38: pituitary adenylate cyclase activating polypeptide-38
PACAP-38-LI: pituitary adenylate cyclase activating polypeptide-38-like immunoreactivity
PACAP-LI: pituitary adenylate cyclase activating polypeptide-like immunoreactivity
RIA: radioimmunoassay
SP: substance P
TFA: trifluoroacetic acid
TNC: trigeminal nucleus caudalis
TRG: trigeminal ganglion
TS: trigeminovascular system
VIP: vasoactive intestinal peptide
$VPAC_1$: VIP, VIP1 or PACAP type II receptor
$VPAC_2$: VIP2 or PACAP type III receptor