INVESTIGATION OF THE ROLE OF SOMATOSTATIN AND PACAP IN MODELS OF INFLAMMATION, PAIN AND TRIGEMINOVASCULAR ACTIVATION

PhD THESIS

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INTRODUCTION

The triple action of capsaicin-sensitive nociceptive nerve terminals

Capsaicin-sensitive, Transient Receptor Potential Vanilloid 1 (TRPV1)-expressing sensory nerve terminals exert three actions: afferent, local and systemic efferent functions. The classical afferent activity is the transmission of nociceptive information into the central nervous system, which leads to nociception and pain sensation. Besides this process, sensory neuropeptides, such as calcitonin gene related peptide (CGRP) and tachykinins (substance P: SP and neurokinin A: NKA) are released from these activated nerve endings, which induce vasodilation and plasma protein extravasation collectively called neurogenic inflammation in the innervated area. These pro-inflammatory actions refer to the local efferent function of capsaicin-sensitive afferents. The third, systemic efferent function of capsaicin-sensitive sensory nerves was discovered earlier by our research group. Several lines of evidence have been provided that besides the locally liberated pro-inflammatory neuropeptides, somatostatin (SST) is also released from the activated afferents, reaches the circulation and exerts systemic anti-inflammatory and analgesic actions. This endogenous counter-regulatory mechanism of sensory nerve-derived somatostatin has been defined as its „sensocrine function" on the basis of its well-known endocrine and paracrine actions.

Sensory neuropeptides released from the activated capsaicin-sensitive nerve terminals

One group of ***pro-inflammatory*** sensory neuropeptides released from stimulated capsaicin-sensititve sensory nerves is called tachykinins, which includes substance P, neurokinin A and neurokinin B. They exert their effects via G protein-coupled neurokinin receptors. SP enhances vascular permeability and results plasma protein extravasation, stimulates the chemotaxis, the proliferation and cytokine production of lymphocytes, activates mast cells and causes neutrophil accumulation. NKA induces smooth muscle contraction, plasma protein extravasation and stimulation of inflammatory cells. NKB binds to NK3 receptor, which is mainly present in the central nervous system and in some peripheral organs. CGRP possesses strong vasodilator action, mainly via CGRP1 receptor activation. CGRP itself does not enhance directly vascular permeability, but potentiates the action of SP.

Anti-inflammatory and anti-nociceptive neuropeptides are somatostatin and Pituitary Adenylate-Cyclase Activating Polypeptide (PACAP), they will be described in more details in the 1st and 3rd sections of the thesis. Opioid peptides are also part of this group, they inhibit immune cell proliferation, chemotaxis, superoxide and cytokine production, as well as mast cell degranulation.

Pain, nociception, hyperalgesia, allodynia

According to the definition of the International Association for the Study of Pain, nociceptive **pain** is a psycho-physiological phenomenon, a subjective sensory quality, with a neurobiological component called **nociception** (perception of painful stimuli, sensory experience), which can be examined in animal experiments. Meanwhile, the affective component (emotional experience of the pain) can be evaluated only in humans. Nociception appropriate for examination in animal experiments is produced in response to mechanical (touch, pressure), thermal (hot or cold temperature) or chemical (capsaicin, formalin, acetic acid, etc.) stimuli. The latency or threshold of the nocifensive reaction can be measured. Increased sensitivity in response to otherwise non-painful stimuli is determined as **allodynia**, while enhanced pain reaction induced by mild noxious stimuli is called **hyperalgesia**

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1. EXAMINATION OF THE ROLE OF THE SOMATOSTATIN SST_4 RECEPTOR IN ACUTE AND CHRONIC INFLAMMATION AND PAIN MODELS

BACKGROUND

Pathophysiological mechanisms of joint inflammation, involvement of neurogenic inflammatory components and the role of neuro-immune interactions

Neurogenic inflammation plays a significant role in the pathophysiological mechanisms of several diseases, e.g. asthma, psoriasis, ekzema, allergic contact dermatitis, inflammatory bowel-and eye diseases, and rheumatoid arthritis (RA). The capsule and the synovial tissue are densely-innervated by capsaicin-sensitive peptidergic sensory fibers. The activation of these nerve terminals mediate stretch and pain, while SP, NKA and CGRP released from them act back on the endings, vessels, synovial and inflammatory cells significantly contributing to the inflammatory reaction, as well as consequent pain and hyperalgesia.

RA is a chronic, progressive polyarticular inflammation with autoimmune background. It leads to pain, disability, and significant deterioration of life quality via articular destruction. Early basis therapy with novel biological agents is the most beneficial treatment and allows the best chance for the patients. They have a direct effect on certain disease processes predominantly via influencing the effects of inflammatory cytokines (TNFα, IL-1β). However, no drugs can effectively inhibit the neurogenic components of inflammatory diseases.

Mechanisms of neuropathic pain and its investigational techniques

Peripheral neuropathy means impairment of the peripheral nervous system related to different origins, with consequent loss of function, pain, and further complications originating from other organ disfunctions.

The three main mechanisms in the background of neuropathy: a) **Waller degeneration**: neural (axon, myelin) and other components (epi-, peri, endoneurium) of the affected nerves are simultaneously impaired. In this case there is only a minimal chance to regain the function, and if so, it occours through a collateral transduction by other nerves („*ephactic crosstalk"*and axonal „*sprouting*"). However this mechanism can lead to further complicatins and dysesthesias due to inappropriate connections. b) **Primary demyelinisation**: the primary factor is myelin injury, and axonal structures are intact at the beginning. In turn, with the loss of saltatoric transduction, there will be an impairment of conduction velocity, or in more serious cases, conduction block develops. c) **Axonal neuropathy** is rare, but has the worst prognosis with primary axonal lesion.

The Seltzer-operation is a widely used model for the investigation of peripheral neuropathic pain in animals. The paper of Zeev Seltzer and collagues described the partial sciatic nerve ligation-induced traumatic neuropathy in rats. The main advantage of this model is the lack of voluntary mutilation and the limb is not insensitive, therefore stimulation-evoked responses with von Frey and Randall Selitto methods can be perfectly investigated. Based on the mixed type of the nerve, sensory, motor and vegetative fibers are impaired in different extent and proportion. On the 7^{th} day, when mechanonociceptive threshold measurements are usually performed, there is no motor incoordination (unpublished data). In addition, this model is technically easy, rapid, involves minimal tissue trauma arising from the operation, has a good reproducibility and reliability. The developing mechanical hyperalgesia/allodynia can be well investigated.

Somatostatin and its receptors, their role in inflammation and nociception

Somatostatin, also known as somatotropin release inhibitory factor (SRIF, SST) is widely distributed throughout the body in 14- and 28 aminoacid-containing forms. SST is present in the central and peripheral nervous systems, the gastrointestinal tract, the pancreas, the lung, the adrenal and thyroid glands, inflammatory cells and gonads. Activated synovial cells in the joint and inflammatory cells also secrete SST, which exerts its effects through autocrine or paracrine mechanisms. SST inhibits the secretion of several hormons, the motility of the gastrointestinal tract, gastric/bowel juice secretion and the proliferation of tumor cells. SST has a potent immuno-modulator action, it decreases the secretion of IgA, IgM, IgE from B-cells, inhibits IL-2, IL-4, IL-10 and interferon-γ (IFNγ) production of T-cells, the chemotaxis of neutrophils, the phagocytic and killer activity of macrophages and natural killer cells. SST possesses neuromodulator function in the central nervous system, inhibits the release of several neurotransmitters (glutamate, serotonine, acetil choline) and neurohormones (GHRH), it influences locomotor activity and modulates cognitive functions, its importance is proved in several neurological and psychiatric diseases. SST is also synthesized and stored in the capsaicin-sensitive, TRPV1 receptor-expressing sensory neurons. Exogenously administered SST alleviates pain in animal models and different pain diseases. It exerts its effects via its own G_i protein–associated receptors. Five somatostatin receptors have been cloned in mice, rats and humans (sst_1, sst_2, sst_3, sst_4 and sst_5). They can be divided into 2 main groups on the basis of sequence similarities and binding of synthetic somatostatin analogues: the SRIF1 group comprises of sst_2, sst_3, and sst_5 receptors, and the SRIF2 group contains sst_1 and sst_4 receptors. Our group has provided several lines of evidence that SRIF2 receptors, predominantly the sst_4, is responsible for the anti-inflammatory and anti-nociceptive actions of SST at the periphery.

Generation of sst_4 receptor gene–deficient mice provided a great opportunity to elucidate the role of these receptors in physiological/pathophysiological conditions. The therapeutic value of native somatostatin is limited by its broad range of effects and short plasma elimination half life. However, potent and stable agonists acting selectively at sst_4/sst_1 receptors on nociceptive nerve terminals and inflammatory cells could be promising for anti-inflammatory and analgesic drug development.

Somatostatin sst_4 receptor-selective agonists

The main advantage of these agonists is the lack of endocrine effects mediated by somatostatin sst_2, sst_3 and sst_5 receptors. The cyclic heptapeptide TT-232, a sst_4/sst_1 receptor agonist molecule has wide range of antinociceptive effects, which is supported by earlier results of our research group.

The present experiments were performed with a high affinity, sst_4-selective agonist, J-2156, which was synthesized at Juvantia Pharma Ltd. (Turku, Finland). J-2156 is a non-peptide sulphonamido-peptidomimetic compound, its exact chemical structure is (1'S,2S)-4-amino-N-(1'-carbamoyl-2'-phenylethyl)-2-(4''-methyl-1''-naphthalene-sulphonylamino)-butanamide.

This molecule has nanomolar binding affinity to the human sst_4, which is greater than that of native somatostatin, and it is at least 400-fold more selective for sst_4 receptor than the other four subtypes.

AIMS

One goal of my PhD work was to investigate the role of sst_4 receptors in acute and chronic inflammation models, as well as in traumatic mononeuropathy model using sst_4 knockout mice and the selective sst_4 receptor agonist J-2156.

MODELS AND METHODS

Animals

Heterozygous mice ($sst_4^{+/-}$) which were generated by the group of Piers Emson (Babraham Institute, Cambridge, UK) on the C57Bl/6 background. They were bred and kept in the Laboratory Animal House of our department under standard conditions at 24-25°C and provided with standard chow and water *ad libitum*. We used male $sst_4^{-/-}$ and $sst_4^{+/+}$ mice from the first 3 inbred generations for the experiments, weighing 20-25 grams. In certain experiments $sst_4^{+/+}$ and $sst_4^{-/-}$ littermates were also tested for comparison.

Experimental models:

- Carrageenan-induced acute paw inflammation

$Sst_4^{+/+}$ and $sst_4^{-/-}$ mice bred as separate lines were tested simultaneously with littermate controls in this model. Carrageenan (3%, 50 µl) was injected intraplantarly into one hindpaw to induce a mixed-type inflammation, containig neurogenic and non-neurogenic components. Separate groups of $sst_4^{+/+}$ and $sst_4^{-/-}$ animals were pretreated with the selective sst_4 receptor agonist J-2156 (100 µg/kg i.p.) 10 minutes before carrageenan administration, saline pretreated animals served as controls.

- Complete Freund's Adjuvant (CFA)-evoked chronic inflammation model

Inflammation of the tibiotarsal joints was evoked by s.c. injection of complete CFA (killed *Mycobacteria* suspended in paraffin oil; 50µl, 1 mg/ml) intraplantarly and s.c. into the root of the tail. In order to enhance the systemic effects, an additional injection was given into the tail the following day, this was considered as the first day of the experiment.

-Traumatic mononeuropathy induced by sciatic nerve ligation

$Sst_4^{+/+}$ and $sst_4^{-/-}$ mice were anaesthesized and the common sciatic nerve was exposed unilaterally high on the thigh, then 1/3-1/2 of the nerve trunk was carefully separated and tightly ligated under an operation microscope. Then the animals were allowed to recover and survive for 7 days. The mechanonociceptive threshold of the hindpaws was determined before and 7 days after surgery (in order to prove the development of hyperalgesia). Both $sst_4^{+/+}$ and $sst_4^{-/-}$ mice were treated with the selective sst_4 agonist J-2156 (100 µg/kg i.p.), 30 minutes later the change of the mechanonociceptive threshold was measured again.

Methods:

-Mechanical touch sensitivity measurement of the paw

The mechanonociceptive threshold was measured by a Dynamic Plantar Aesthesiometer, which is a modified electronic von Frey device. Mice move freely within the cage on the metal mesh surface. With the help of a blunt end needle the operator stimulates the planar surface of one hindpaw with an increasing force. When the mechanonociceptive threshold is reached, the animal withdraws its hindpaw and the counter stops. The paw withdrawal threshold is numerically shown in grams on the digital screen. Hyperalgesia in response to the inflammation or the neuropathy was expressed in percentage compared to the initial control values.

-Measurement of the paw volume

The volume of the paw was measured by plethysmometry. This works by detecting fluid displacement on the basis of communicating vessels when dipping the paw into the special fluid. The volume can be read in cm^3 from the digital screen.

-Determination of spontaneous weight distribution

In order to investigate the weight spontaneously distributing on the two hindpaws we used an incapacitance tester. The basic part of this equipment contains two scales positioned paralelly. In response to inflammation of one limb, the animals try to avoid pain and much less weight is distributed on the affected side.

-Examination of inflammatory cytokines with ELISA and Cytometric Bead Array (CBA) methods

1. Measurement of IL-1β concentration of the joint homogenates

IL-1β concentration of the joint homogenates was determined by an OptEIA ELISA set. Data were expressed as pg cytokine/g wet tissue.

2. Measurement of TNFα, IL-2, IL-4, IL-5 and IFNγ concentration of the joint homogenates

The concentration of cytokines mentioned above was determined by a Mouse Th1/Th2 cytokine Cytometric Bead Array (CBA) kit.

RESULTS

-The role of sst_4 receptors in carrageenan-induced acute inflammation

The control paw volumes and mechanonociceptive thresholds of $sst_4^{+/+}$ and $sst_4^{-/-}$ mice did not differ significantly. Carrageenan administration caused inflammation of the treated paw with a marked swelling, redness, and decrease of the mechanonociceptive threshold. Six hours after the injection both oedema and the drop of the nociceptive threshold were significantly greater in the knockout group. The results of wildtype and sst_4 receptor-deficient littermates produced from heterozygote mice were essentially the same as data obtained in $sst_4^{+/+}$ and $sst_4^{-/-}$ mice bred as separate lines. Pretreatment with the selective peptidomimetic sst_4 receptor agonist J-2156 significantly inhibited mechanical hyperalgesia in wildtype mice, but not in the knockouts. Surprisingly, J-2156 did not inhibit paw swelling in either group.

-The role of sst_4 receptors in adjuvant-induced chronic inflammation

Inflammatory mechanical hyperalgesia was greater in $sst_4^{-/-}$ mice than in their wildtype counterparts throughout the whole 21-day experimental period, but the difference was more pronounced and shown to be significant only in 4 timepoints of the study.

In the first 12 days of the study significantly less weight was distributed on the treated hindlimb in the $sst_4^{-/-}$ than in the $sst_4^{+/+}$ group, but no difference could be observed later. There was significant difference of paw swelling between the two groups at the 4^{th} and 6^{th} days- paw swelling was between 80% and 100% in the later period.

The concentration of inflammatory cytokines IL-2, -4 és -5 were negligible in both groups. We found significantly greater amount of IFNγ and TNFα in the knockout group, but the concentration of IL-1β did not differ in the wildtype and knockout groups.

-The role of sst_4 receptor in traumatic mononeuropathy

Seven days after partial ligation of the sciatic nerve 31-34% mechanical hyperalgesia developed both in $sst_4^{+/+}$ and $sst_4^{-/-}$ mice. The sst_4 selective agonist J-2156 was ineffective in both groups.

DISCUSSION

The present data provide clear evidence for the protective role of sst_4 receptor in acute and chronic inflammatory processes. Our group has shown earlier that exogenous administration of somatostatin and synthetic agonists (TT-232 and J-2156) diminish neurogenic vasodilatation, plasma protein extravasation, and the release of substance P and CGRP from nerve endings.

We found significantly greater mechanical hyperalgesia in both inflammation models, and significantly greater paw oedema in the early phase of the acute model in case of $sst_4^{-/-}$ animals. In addition, in case of chronic inflammation, the concentrations of IFNγ and TNFα were significantly higher in the knockout group, and the weight spontaneously distributed on the affected limb was also less.

Endogenous activation of the sst_4 receptor results in pronounced analgesic and anti-oedema effects in the early phase of chronic inflammation, but this effect cannot be seen in the later phase. The accumulated macrophages and lymphocytes in the inflamed area can release further mediators, which migh counteract the anti-inflammatory effect of SST. This could be an explanation for the disappearence of the protective role of sst_4 receptors in the late inflammatory phase

The selective sst_4 agonist J-2156 diminished acute inflammatory mechanical hyperalgesia, allowing further confirmation for the anti-nociceptive and anti-inflammatory actions of this receptor activation. In contrast to results obtained in inflammatory models, traumatic mononeuropathy-evoked mechanical hyperalgesia was not diminished either by the lack of sst_4 or its exogenous activation by J-2156. It is surprising, because or group has reported earlier the anti-hyperalgetic action of this agonist in the same neuropathy model of the rat. Differences in the mechanisms of neuropathy between mice and rats, distinct localization/density of sst_4 receptors, or different kinetic parameters of J-2156 can serve as explanations for these contradictory results. J-2156 in previous experiments exerted effective inhibitory actions on a variety of inflammatory processes in rats by decreasing the release of SP and CGRP from sensory nerve terminals and also by directly inhibiting vasodilatation and immune cell functions. Since J-2156 is a selective sst_4 receptor agonist, it does not induce endocrine effects mediated by the sst_2, sst_3 and sst_5 somatostatin receptors, e.g. inhibition of GH, glucagon, insulin release. Based on these results J-2156 might have a great importance fom drug development.

Although little is known about the localization of sst_4, data obtained with the selective agonist suggest that the inhibitory actions observed by us take place at the levels of the sensory nerve terminals, vascular endothel cells and inflammatory cells. This latter effect was proved *in vitro* by the inhibition of IL-1β production of isolated peritoneal macrophages.

2. COMPARISON OF THE ANTI-INFLAMMATORY AND ANTI-NOCICEPTIVE EFFECTS OF CORTISTATIN-14 AND SOMATOSTATIN-14 IN DISTINCT IN VIVO AND IN VITRO MODEL SYSTEMS

BACKGROUND

Cortistatin and its role in nociceptive and inflammatory processes

Cortistatin (CST) is structurally closely related to SST and got this name after its predominantly cortical expression and ability to depress cortical activity. Similarly to SST, CST occurs in 14 or 17 aminoacid-containing forms in rat, mouse, and human tissues, but it is also present in a longer form of 29 aminoacids in rats and humans.

CST-14 shares 11 of 14 aminoacids with SST-14, including two cysteine residues that are likely to make the peptide cyclic and the FWKT motif (Phe, Trp, Lys, Thr) that is critical for sst receptor binding. It has been shown that CST is able to bind to all the five sst receptors and shares many pharmacological and functional properties with SST including the inhibition of neuronal activity.

Intracerebroventricular injection of CST decreased locomotor activity, whereas higher doses of CST (10 μg) evoked seizures, a phenomenon which is also observed following higher doses of SST. It is possible that CST is able to exert its effects *in vivo* via sst receptors. A distinct biological activity of CST compared to SST is the induction of slow-wave sleep, presumably by antagonizing the excitatory effects of acetylcholine on the cortex.

The expression of CST mRNA follows a circadian rhythm and it is upregulated in response to sleep deprivation, suggesting that CST is a sleep-modulating factor. In contrast to SST, endogenous CST stimulates the secretion of prolactin, the prolactin level in the circulation of CST knockout mice is significantly lower. Recent data showed a protective role of CST against vascular calcification in rats, which is likely to happen via GHS-R1 receptor.

CST-14 is a very potent anti-inflammatory peptide, since it inhibits the proliferation of Th1 cells and the release of pro-inflammatory cytokines (IL-2 and IFNɣ), while increases anti-inflammatory signals (IL-10). Since there are distinct biological activities of CST compared to SST, the existence of CST-specific receptors can be strongly suggested. Although the inhibitory actions of CST on the cellular components of inflammatory processes have been extensively investigated and well described, little was known about its effects on sensory nerves, neurogenic inflammation and hyperalgesia.

AIMS

Our aim was to examine the effects of CST in comparison with SST on inflammatory mechanisms with special emphasis on the neurogenic factors in systematically designed *in vitro* and *in vivo* model systems.

MATERIALS AND METHODS

Animals

In vivo experiments were performed on male CD1 mice (25-30 g) and Wistar rats (150-250 g). *In vitro* experiments were done on tracheae dissected from Wistar rats. The animals were

bred and kept under standard conditions at 24-25°C and provided with standard rodent chow and water *ad libitum*.

-Competition binding analysis

CHO cells stably expressing sst_4 receptors (CHO-sst_4) and sst_1 receptors (CHO-sst_1) were produced by us and analyzed in competition binding assays. Confluent cells on 24-well plates were incubated with 1 ml buffer containing 1 mg/ml bacitracin and 10 nM [^{125}I-Tyr^{11}]SST-14 (labeled by us with the ^{125}I isotope) for 30 min at room temperature. In the same time, cells were incubated with increasing concentrations of CST-14 (10^{-9}- 10^{-5} M), washed after 30 minutes and lysated. Radioactivity of the solution containing the lysate was measured with a γ-counter.

- G-protein activation assay: [^{35}S]GTPγS binding test

Membrane fractions were prepared from CHO-sst_4 and CHO-sst_1 cells in Tris–EGTA buffer. Membrane fractions were incubated at 30 °C for 60 min in the same Tris–EGTA buffer containing [^{35}S]GTPγS (0.05 nM) and increasing concentrations (10^{-9} to $10^{-5.5}$ M) of SST-14 and CST-14 in the presence of 30 μM GDP. The reaction was terminated by filtrating the samples through Whatman GF/B glass fiber filters. Non-specific binding was determined in the presence of 10 μM unlabelled GTPγS. After drying for 60 min at 37°C radioactivity was measured in scintillation liquid in a Packard Tri-Carb 2800 TR scintillation counter. SST-14 and CST-induced G-protein activation was given as percentage over the specific [^{35}S]GTPγS binding observed in the absence of the agonists.

- Examination of interleukin-1β synthesis of stimulated peritoneal macrophages

Salmonella typhimurium endotoxin (LPS; 300 μg/ml, 300 μl,) was injected i.p. to male CD1 mice. Four hours later mice were exsanguinated under deep anaesthesia, then the abdominal cavity was washed with ice-cold cell culture medium to collect inflammatory cells. Then the cell culture medium was added to the samples of the lavage fluid and 1 μg/ml LPS was used to further stimulate the macrophages. In control experiments saline, SST-14 or CST-14 solutions were administered to each well. The plates were then incubated for 8 h under standard conditions. Then the contents of the wells were collected, centrifuged and the cells were identified by flow cytometry. The concentration of the inflammatory cytokine IL-1β was measured by sandwich ELISA technique.

- Measurement of capsaicin-evoked sensory neuropeptide release from isolated rat tracheae

Rats were exsanguinated in deep anaesthesia, then the whole tracheae were removed and placed into an organ bath to be perfused with pH 7.2 controlled, oxygenated Krebs solution for 60 min (equilibration period) at 37°C. After discontinuation of the flow, the solution was changed three times for 8 min to produce prestimulated, stimulated, and poststimulated fractions. Capsaicin-evoked stimulation (10^{-6} M) was performed to elicit neurotransmitter release in the second 8-min period. CGRP concentrations were determined from 200 μl samples of the organ fluid of the preparations by means of radioimmunoassay methods developed in our laboratories, as described. Saline, SST-14 or CST-14 was added to the incubation medium at the beginning of each fraction. Both compounds were administered in a concentration range of 10–2000 nM in separate experiments, only one concentration was applied to the same tracheae to avoid neuropeptide depletion.

- ***Examination of mustard oil-induced acute neurogenic inflammation in the dorsal skin of the rat hindpaw***

Both hindlegs of the rats were acutely denervated (the sciatic and the saphenous nerves were cut 30 min before the induction of the inflammation) under anaesthesia to avoid central

reflexes. Acute neurogenic inflammation in the paw skin was evoked by topical application of 1 % mustard oil. Extravasation of plasma albumin was measured by the Evans blue leakage method. Evans blue (50 mg/kg) was injected i.v. and neurogenic inflammation was induced 10 min later. Rats were killed by exsanguination 20 min after mustard oil application. The skin of the hindpaws was removed and the extravasated dye was extracted with formamide for 72 h at room temperature for photometric determination with a microplate reader. The amount of the accumulated Evans blue was expressed as μg dye/g wet tissue. CST-14, SST-14 or in the control group the same volume of saline was administered i.p. 20 min before the induction of the inflammation.

- ***Measurement of carrageenan-induced mechanical hyperalgesia and oedema formation of the mouse paw, as well as inflammatory cytokine production in the plantar skin homogenates***

The polysaccharide carrageenan was injected (3%, 50 μl) into the plantar surface of one hindpaw of male CD1 mice under anaesthesia to induce a mixed-type inflammatory response with both neurogenic and non-neurogenic components. SST-14, CST-14 (100 μg/kg i.p.), or in the control group saline was administered 15 min before the induction of the inflammation and before each measurement, which were performed 1, 2, 4, 6, and 23 hours after carrageenan administration in the case of mechanonociceptive threshold and 6 hours after carrageenan administration in the case of plethysmometry. The mechanonociceptive thresholds were measured with aesthesiometry, and the volume of the paw with plethysmometry. 24 hours after the induction of the inflammation mice were anaesthetized and then sacrificed. The plantar skin samples were excised, weighed, frozen in liquid nitrogen and kept at -80°C until further processing. The presence of two inflammatory cytokines, IL-1β and TNFα were determined by sandwich ELISA techniques.

- Investigation of mild heat injury-induced thermal hyperalgesia of the rat paw

The noxious heat threshold of the paw of male Wistar rats was determined with a validated increasing-temperature water bath, which was developed in our laboratory in cooperation with Experimetria Ltd. (Budapest, Hungary). A starting temperature of 30 °C was employed and the cut-off temperature was set to 53 °C. After control threshold measurements rats were anaesthetized with diethyl ether and one of the hindpaws was immersed in a constant, 51°C hot water bath for 20 sec in order to evoke a mild heat injury. Following recovery from anaesthesia, heat threshold determinations were repeated 20 min after the injury to confirm the development of hyperalgesia. SST-14, CST-14 or in the control group saline was administered i.p. (100 μg/kg) in one group and i.pl. in the other one (100 μl; 250 μg/ml solution) directly after the 20-min measurement, then the heat thresholds were measured repeatedly every 20 min for 80 min.

RESULTS

-Binding of cortistatin to sst_1 and sst_4 receptors

CST-14 applied in increasing concentrations (10^{-9} to 10^{-5} M) displaced the 10 nM radiolabeled SST-14 from both the CHO-sst_4 and CHO-sst_1 cells in a concentration-dependent manner. The maximum of the inhibition was observed using 10^{-6} M cortistatin (98.4%) on CHO-sst_4 cells, and at the same concentration (83.6%) on CHO-sst_1 cells. The IC_{50} value was 12 nM and 18 nM in the case of CHO-sst_4 and CHO-sst_1 cells, respectively, showing similar affinities of CST-14 and SST-14 to these receptors.

-G-protein activation by cortistatin and somatostatin

Increasing concentrations of both peptides resulted in a concentration-dependent stimulation of [^{35}S]GTPγS binding. EC_{50} values were defined as the concentration of the ligands producing 50% of their maximal response. In the case of SST-14 this value was 10 nM and 13 nM on the CHO-sst_4 and CHO-sst_1 cell lines, respectively. The EC_{50} values of CST-14 were 33 nM and 28 nM in cases of the CHO-sst_4 and CHO-sst_1 cells. The maximal activation values represent the percentage stimulation of [^{35}S]GTPɣS binding over basal activity. In case of SST-14 these data were 218% and 204% the CHO-sst_4 and CHO-sst_1 cell lines, respectively, while for CST-14 the corresponding values were 223% and 218% showing similar efficacies for the two peptides.

-The effect of somatostatin and cortistatin on IL-1β synthesis of stimulated peritoneal macrophages

The concentration of IL-1β in the culture media of LPS-treated isolated murine peritoneal macrophages was 473.76+38.81 pg/ml in case of control experiments after the addition of 100 µl saline. The amount of this inflammatory cytokine after LPS stimulation was not altered in the presence of 0.1 µg/ml SST-14 or CST-14. However, the two higher concentrations, 1 µg/ml and 10 µg/ml SST-14, as well as CST-14 induced significantly lower cytokine production: SST-14 evoked an about 33-35% decrease, while the inhibitory effect of the same concentrations of CST-14 was significantly greater, 70-75%. Concentration-response correlations were not observed in this concentration range.

- The effects of SST and CST on capsaicin-evoked sensory neuropeptide release from isolated rat trachea

Both SST-14 and CST-14 significantly inhibited the stimulation-evoked CGRP release, but concentration-response correlation was not observed in either case, the maximal inhibition was produced by the lowest SST concentration. The inhibitory effects of 2 µM CST-14 and 100 nM SST-14 were similar, 85% and 87%, respectively. These can be considered as the maximal inhibitory actions, both peptides exerted smaller effects in higher concentrations. Neither compound influenced the basal, non-stimulated peptide outflow.

-The effects of SST and CST on mustard oil-induced acut neurogenic inflammation in the dorsal skin of the rat hindpaw

Both SST-14 and CST-14 significantly decreased mustard oil-induced cutaneous plasma protein extravasation detected 20 min after topical application of the inflammatory stimulus. SST-14 induced an about 60% inhibitory effect, while CST-14 exerted a remarkably greater, almost 80% inhibition.

-The effects of SST and CST on carrageenan-induced mechanical hyperalgesia, paw oedema and inflammatory cytokine production

Remarkable mechanical hyperalgesia and paw swelling developed in the control, saline-treated group in response to i.pl. 3% carrageenan injection reaching a maximal hyperalgesia of 40% and oedema of 55% at the 6-hour timepoint. Treatment with the 100 µg/kg i.p. dose of SST-14 showed an inhibitory tendency, which only proved to be significant at the 4 h timepoint. However, in the CST-14-treated group hyperalgesia was significantly smaller at all measurement points from 2 hours to 23 hours than in saline-treated mice. Meanwhile, the extent of the oedema formation was similarly decreased in both groups by 30-35% at the 6 h timepoint. Carrageenan injection induced a remarkable IL-1β and TNFα production in the plantar skin of saline-treated control mice. Repeated i.p. injections of 100 µg/kg both SST-14 and CST-14 significantly decreased IL-1β synthesis, but only CST-14 reduced TNFα concentrations significantly.

-The effects of SST and CST on mild heat injury-evoked thermal hyperalgesia in rat hindpaw

After the heat injury, rats recovered from anaesthesia within minutes. There were no signs of spontaneous nocifensive behavior in any of the animals. About 5-6°C drop of the thermonociceptive threshold was observed 20 min after the injury, and this heat hyperalgesia was maintained at this level for the 80 minutes of the measurement. Intraperitoneal, as well as intraplantar administrations of both SST-14 and CST-14 directly after the 20-min measurement, similarly and significantly decreased heat hyperalgesia throughout the whole 80-min experimental period as compared to the solvent-treated groups, although the inhibitory action of i.p. CST-14 was not statistically significant at the earliest, 40-min timepoint.

DISCUSSION

The result of the present comparative study demonstrated that the two structurally closely related peptides, CST and SST, have similar binding profile and agonistic behavior to the sst_1- and sst_4 somatostatin receptors that we have previously shown to be responsible for the anti-inflammatory and anti-nociceptive actions of somatostatin. The major novelty of this series of experiments is that we provided clear *in vitro* and in *vivo* evidence that these peptides have potent inhibitory action on the release of the pro-inflammatory sensory neuropeptide CGRP from peripheral sensory nerve terminals, consequent acute plasma protein extravasation, heat and mechanical hyperalgesia. A very recent paper demonstrating the inhibitory action of CST and SST on chemical stimulation-induced CGRP release in primary cultures of rat trigeminal neurones supports the results we got at the level of the peripheral nerve endings. Despite similar actions of CST and SST on the neurogenic inflammatory parameters and hyperalgesia, CST-14 exerts significantly greater inhibitory effect on the cellular inflammatory factors, as shown by a more pronounced attenuation of endotoxin-stimulated IL-1β production of cultured murine peritoneal macrophages and also by significantly decreased carrageenan-evoked TNFα synthesis in the plantar skin of the mouse. Carrageenin-induced oedema and mechanical hyperalgesia related to both neurogenic and non-neurogenic inflammatory mechanisms are similarly diminished by both peptides.

In addition to their only partially overlapping expression pattern, several similar, but also different functions have already been described. CST was originally shown to have sleep promoting properties and effects on animal locomotor behavior, which are in contrast to those reported for SST. This proposed the existence of own cortistatin receptors, and/or the ability of CST to act at other targets, such as the orphan receptor MrgX2 (Mas-related gene) which is related to the *Mas1* oncogene. Tissue distribution studies have shown that these receptors are expressed mainly in small sensory neurons of the dorsal root ganglia (DRG) suggesting their role in nociception. However, quantitative PCR studies revealed much broader expression of CST than that of MrgX2 receptors. Unlike SST, CST also shows high affinity to ghrelin receptor (GHS-R1a) which has been suggested to be involved in its anti-inflammatory actions. Furthermore, similarly to morphine and methadone at μ receptors, differences in receptor internalization may also be responsible for the observed differences between the physiological effects of CST-14 and SST-14. It has been shown that CST mRNA expression is not increased in SST-deficient mice, which suggests that the role of CST is likely not to be a SST back-up.

Since we show here a similar abilty of CST to diminish acute neurogenic inflammation and hyperalgesia, the involvement of sst_4 and sst_1 receptor activation on the sensory nerve terminals can be proposed in these actions as well. Meanwhile, the inhibitory effect on immune cell functions is possible to be related to sst_2 activation. Furthermore, a participation

of sst_2, sst_3 and sst_5 receptors in the analgesic actions might also be possible on the basis of the results provided by others. The concept of the peripheral analgesic mechanism of action is supported by the finding that both peptides similarly decreased heat injury-induced acute thermal hyperalgesia after systemic peripheral and also local administrations. Since a rapid sensitization of the peripheral nerve endings plays a predominant role in this type of heat hyperalgesia, these anti-hyperalgesic actions are likely to be independent of the anti-inflammatory effects. Besides the peripheral actions, CST is likely to be involved in central pain processing pathways too, since its intracerebroventricular administration elevated the latency of nocifensive behaviors in response to thermal stimulation.

In contrast to the neurogenic mechanisms, the actions of CST on inflammatory and immune cells are well described in several experimental systems. CST prevents sepsis-induced mortality by inhibiting the production of inflammatory mediators by activated macrophages and decreasing the recruitment of neutrophils and monocytes to inflamed organs. Furthermore, CST inhibits macrophage functions by downregulating the production of a broad range of inflammatory mediators. Deactivation of resident and infiltrating macrophages is a major mechanism involved in the anti-inflammatory action of CST in collagen-induced arthritis. CST was significantly more efficient than SST to inhibit this chronic joint inflammation, which also supports the idea that it might have capacity to activate different receptors and transduction pathways.

Our data supports the results of others showing that CST inhibits the production of inflammatory molecules, such as TNFα, IL-6 and nitric oxide by endotoxin-activated murine peritoneal macrophages. CST showed greater inhibitory effect on the production of these inflammatory mediators than SST or the synthetic sst_2, sst_3 and sst_5 agonist octreotide. Although the non-selective somatostatin receptor antagonist cyclo-somatostatin blocked the effect of SST, it only partially inhibited the actions of CST suggesting that it might act through other receptors being important in decreasing inflammatory cell functions.

In conlcusion, we showed that CST is able to diminish acute neurogenic inflammation, thermal and mechanical hyperalgesia, in which the inhibition of pro-inflammatory sensory neuropeptide release from the peripheral (but presumably also from the central) terminals is likely to play an important role. Since these effects are very similar to those of SST, the involvement of somatostatin receptor activation is proposed. In contrast, CST exerted markedly greater inhibitory action on inflammatory cytokine production than SST, which is likely to occur trough sst-independent mechanisms.

3. THE ROLE OF PACAP IN TRIGEMINOVASCULAR ACTIVATION

BACKGROUND

PACAP and its role in nociception

Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) is a member of the vasoactive intestinal polypeptide (VIP)/secretin/glucagon peptide family, which is present in 27 and 38 aminoacid-containing biologically active forms. The highest concentrations of PACAP in rats are in the hypothalamus and testicles. It is a sensory neuropeptide localized in the dorsal horn of the spinal cord, dorsal root ganglia, as well as in the peripheral terminals of capsaicin-sensitive sensory nerves. It is also present in afferents innervating the joint capsule, and in the central nervous system. PACAP-immunoreactivity was also shown in the cell bodies and nerve fibres of "migraine generator" region of the human brainstem, the trigeminal nucleus caudalis and the C1-C2 segments of the cervical spinal cord. Brain mast cells, which are located perivascularly in the dura in close association with neurons also contain PACAP. They can be activated following trigeminal nerve activation, cervical or sphenopalatine ganglion stimulation.

PACAP have several actions in the body: it controls the release of neurotransmitters, induces vasodilatation, bronchodilatation, enhances the bowel motility, increases the concentration of certain hormones. These effects of PACAP are mediated via G protein-coupled receptors named PAC1 and VPAC1/VPAC2. The PAC1 receptor binds specifically PACAP whereas VPAC1/VPAC2 have a similar binding affinity for VIP and PACAP. All these receptors have been described on neurons, smooth muscle cells, mast cells and several inflammatory cells, their activation is associated with the adenylate cyclase and phospholipase C signal transduction pathways.

Intrathecal administration of PACAP inhibited nociceptive reflexes and inflammation induced nociception. Others also found that central application of PACAP dose-dependently decreased thermal stimulation-evoked paw withdrawal latencies and potentiated nociceptive transmission to the spinal dorsal horn. In cats, intracerebral microinjection of PACAP resulted in a moderate increase of the cerebral blood flow. Intracerebroventricular application of PACAP exerted analgetic actions in the early phase of formalin test, whereas it was pronociceptive in the late phase of this test. Our group has recently described a peripheral anti-nociceptive, but central pro-nociceptive action for this peptide.

Although the presence of PACAP in several brain regions of humans and animals related to the trigeminal system was described a decade ago, its potential role in trigeminovascular activation has recently been proposed on the basis of the localization of PACAP and PAC1 receptors in the smooth muscle of rat and human intracranial vasculature. Remarkably, it has recently been reported that in migraineurs PACAP infusion triggered migraine-like headache without aura. On the basis of these novel human observations, it has been proposed that PACAP might be a mediator of trigeminovascular activation which plays an important role in migraine.

Trigeminovascular activation and its role in migraine

Migraine belongs to the group of primary neurovascular headaches. The main feature of neurovascular headaches is the tight association between the pain and changes of the intracranial vasculature. Since these are neural changes, we can talk about neurovascular events. Experimental cranial pain induces vasodilation, this reflex also exists in cats, monkeys, and humans. Activation of the first division of the trigeminal nerve increases blood flow in the areas innervated by the first division, both on the face and in the brain.

There are branches of unmyelinated fibers surrounding the large cerebral vessels, dura mater, pial vessels and large venous sinuses, originating from the ophtalmic division of the trigeminal ganglion and the upper cervical dorsal roots. Trigeminal fibers innervating cerebral vessels arise from the trigeminal ganglion containing SP and CGRP. Both peptides are released upon trigeminal ganglion stimulation. Stimulation of cranial vessels-e.g. *superior sagittal sinus* – provokes pain in man. Whatever function can be connected to sterile inflammation of the dura mater in the pathophysiological mechanisms of migraine, it is sure that some form of trigeminal sensitization plays an important role in this process. There could be a peripheral sensitization via the local release of inflammatory mediators, probably resulting in a pathologic activation of the trigeminal nociceptors. The central form of sensitization is, however, more likely in migraine, which can be related to a hypersensitivity of the trigeminal nucleus or dysfunction of the descending pain-modulating pathways.
Neuropeptides also play an important role in the pathological mechanisms of migraine. Electrical stimulation of the trigeminal ganlion leads to an increased extracerebral blood perfusion and local release of SP and CGRP in cats and humans. The human evidence that the level of CGRP is elevated in the headache phase of migraine, supports the theory that activation of the trigeminovascular system has a great importance in these conditions. Besides CGRP, PACAP can be also detected in several brain regions of man and animals associated with the trigeminal system.

AIMS

The aim of our work was to examine the role of PACAP in chemically-induced trigeminovascular activation using behavioral, vascular imaging and morphological techniques with the help of PACAP gene-deleted mice. Although there is no perfect animal model, which could presisely correspond to the human disease, our experiments are in strong correlation with the pathophysiological processes of migraine.

MATERIALS AND METHODS

Animals

Experiments were performed on PACAP gene-deleted ($PACAP^{-/-}$) mice and their wildtype ($PACAP^{+/+}$) counterparts weighing 25-30 g. Knockout mice were generated by Prof. Akemichi Baba and colleagues (Osaka University, Japan). Heterozygous breeding pairs were transported to the University of Pecs. Offsprings within the first three generations were used for the experiments to minimize genetic variations. Mice were kept at 24-25 °C and provided with standard rodent chow and water *ad libitum*.

Experimental model:

-Chemical activation of the trigeminovascular system

Activation of the trigeminovascular system was evoked by the i.p. injection of 10 mg/kg nitroglycerol (NTG), which is a nitric oxide donor (Nitrolingual^{R}).

Investigation methods:

-Investigation of light-aversive behavior

Light-aversive behavior was examined in mice both in the early (0-30 min) and late phases (90-120 min) after NTG injection. A modified light-dark box was used, which has two equally sized compartments, one brightly lit (1000 lux), painted white and lacking a top, the other not lit, painted black and fully enclosed. A small opening connects the two compartments. Time spent in the light phase of the box was measured in each 5-min period and expressed in s

-Determination of meningeal blood flow by laser Doppler blood perfusion scanning

Meningeal microcirculation was determined in urethane-anaesthesized mice with a laser Doppler blood perfusion scanner through the closed cranium. After control images, 10 mg/kg NTG was injected i.p. and the detection lasted for 4 hours to be able to analyze both the early and the late vascular reactions due to the direct vascular effect of NO, as well as the peripheral and central sensitization of the trigeminovascular system, respectively. In case of i.p. PACAP injection, the observation period lasted for 30 minutes. Meningeal blood flow was evaluated by comparing the mean microcirculation values obtained within a respective region of interest of the total cranial area to the mean of the initial control images.

-Examination of neural activation with c-Fos immunohistochemistry

$PACAP^{-/-}$ and $PACAP^{+/+}$ mice deeply anaesthesized with urethane were used for histological purposes 2 and 4 hours following NTG (or in control studies saline) injection. They were perfused transcardially, then brains with the rostral cervical spinal cord and trigeminal ganglia were dissected and post-fixed. To study the caudal division of the spinal trigeminal nucleus, the medulla oblongata caudal to the obex with rostral cervical spinal cord was embedded. Three series of 20 μm coronal sections were cut per animal. Sections were incubated in a polyclonal antiserum raised against c-Fos (sc-52; 1:500). After this, sections were treated with biotinylated goat anti-rabbit IgG (1:200, Vectastain Elite ABC Kit,). After incubation with avidin-biotin complex, development was performed with the help of 0.05% DAB and H_2O_2.
The trigeminal ganglion was sectioned from paraffin embedded samples. Three series of five 4 μm thick longitudinal sections were collected. The first series of sections were used for c-Fos immunocytochemistry following the protocol mentioned above. Digital images were taken (Nikon Eclipse 80i microscope CCD camera) and corrected to obtain optimal contrast, using Photoshop 7.0.1 (Adobe, San Jose, CA). The contents of c-Fos in the TRG and the TNC were determined by simple manual counting all c-Fos-positive cell nuclei in non-edited digital images of 5 serial sections per animal.

- Examination of PAC1 receptor expression with immunohistochemistry

Five sequential trigeminal ganglia and caudal nuclar sections per animal prepared for the c-Fos immunohistochemistry described above were used for PAC1 receptor immunohistochemistry. Sections were treated with the primary antibody anti-PAC1 receptor (raised in rabbit, 1:100), the secondary antibody was Alexa Fluor "568" (1:1000). At least 10 sections were examined from each group. Negative controls with the absence of the primary antisera were done from every group, specific cellular staining was not detected in these cases. For qualitative purposes digital images were adjusted to obtain optimal contrast using Photoshop 7.0.1.

- Exogenous administration of PACAP-38

PACAP-38 was administered through two different ways: in one group of both $PACAP^{+/+}$ and $PACAP^{-/-}$ mice, it was injected i.p. (300 μg/kg) to investigate light-aversive behavior, meningeal blood flow and c-Fos expression 4 h later. In another group, PACAP-38 was administered intracerebroventricularly in deep, long lasting urethane anaesthesia and c-Fos expression was investigated 4 h afterwards. In both cases respective saline-treated mice served as controls.

RESULTS

-Nitroglycerol-induced light-aversive behavior in PACAP$^{+/+}$ and PACAP$^{-/-}$ mice

NTG-treated PACAP$^{+/+}$ animals spent significantly less time in the light side of the box from the 20-minute timepoint providing evidence for the development of photophobia. In contrast, PACAP$^{-/-}$ mice did not show light-aversive behavior in response to NTG injection. Similarly to the early phase, in the late period (90-120 min) NTG-treated PACAP$^{-/-}$ mice spent remarkably longer time in the light side of the box, particularly in the last 10 minutes showing the lack of ligh aversion.

-Nitroglycerol-induced vasodilation on the brain surface of PACAP$^{+/+}$ and PACAP$^{-/-}$ mice

Laser Doppler scanning measurements revealed a gradual increase of meningeal blood flow in PACAP$^{+/+}$ mice 2 hours after NTG injection. This vasodilation remained relatively stable and only started to slightly decrease in the last 15 minutes. In contrast, in the PACAP$^{-/-}$ group there was no change or even a minimal decrease in the meningeal blood flow in response to NTG in the first 2 hours, and its hyperaemic effect was significantly smaller in the second 2-hour period than in PACAP$^{+/+}$ mice. The mean blood pressure decreased by 30-35% in response to this 10 mg/kg i.p. NTG dose under urethane anaesthesia in both groups.

-Nitroglycerol-induced c-Fos expression in the TRG and TNC of nitroglycerol-treated PACAP$^{+/+}$ and PACAP$^{-/-}$ mice

Two hours after NTG injection there was no change in c-Fos expression in the TRG in either group compared to respective saline injected controls. Four hours following this chemical stimulation an about 2-fold increase developed in the TRG of PACAP$^{+/+}$ mice, but it was not altered in PACAP$^{-/-}$ ones. In the TNC of PACAP$^{+/+}$ animals c-Fos immunopositivity was more than double compared to the saline-injected control group already 2 hours following NTG administration, but increased almost to the triple 4 hours later. In contrast, c-Fos expression was unaltered at both time-points in PACAP$^{-/-}$ mice.

-PAC1 receptor expression in the TRG and TNC of PACAP$^{+/+}$ and PACAP$^{-/-}$ mice

Immunofluorescent staining revealed that both the spinal trigeminal nucleus and the trigeminal ganglion neurons express the PAC1 receptor similarly in both groups. The quantification of the PAC1 receptor immunosignal with two-way ANOVA revealed no significant difference in the TRG regarding genotype, NTG treatment, or their interaction. In accordance with these in the TNC, neither genotype and NTG treatment, nor their interaction influenced the receptor expression significantly. This suggests that genotype or NTG stimulation do not influence PAC1 receptor expression in these areas.

-PACAP-38-evoked light-aversive behavior in PACAP$^{+/+}$ and PACAP$^{-/-}$ mice

Administration of 300 μg/kg i.p. PACAP-38 induced remarkable light-aversive behavior in PACAP$^{+/+}$ mice both in the early and late phases of the 2-hour observation period. The pattern of this photophobia was very similar to the effect of NTG. Photophobia did not occur in the PACAP$^{-/-}$ group in response to i.p. PACAP-38 injection.

-PACAP-38-evoked meningeal vasodilation in PACAP$^{+/+}$ and PACAP$^{-/-}$ mice

In PACAP$^{+/+}$ mice i.p. injection of 300 μg/kg PACAP-38 evoked a 20% vasodilation on the brain surface. The same treatment in the PACAP$^{-/-}$ group did not alter meningeal microcirculation during 15 minutes, and only slightly elevated up to a maximum of 10% in the second 15-min period. This response was significantly smaller than that observed in the PACAP$^{+/+}$ animals.

Systemic blood pressure decreased from 80.6±0.4 mmHg to 62.8± 0.1 mmHg within 10 minutes and remained relatively unchanged during the 30-min measurement. No significant difference was observed between the two groups.

-PACAP-38-induced c-Fos expression in the TRG and TNC of PACAP$^{+/+}$ and PACAP$^{-/-}$ mice

I.p. injected PACAP-38 significantly increased neural activation 4 hours later as shown by the elevated number of c-Fos-expressing cells in the TRG of both groups, but only in the TNC of the PACAP$^{+/+}$ mice. In contrast, i.c.v. administered PACAP-38 did not elevate the number of c-Fos-positive neurons in either region in either group. The interesting observations, however, are a) the remarkably increased c-Fos expression in both regions compared to the i.p. injections in PACAP$^{+/+}$ animals even after saline administration, and b) the significantly reduced c-Fos immunopositivity in the TRG of PACAP$^{-/-}$ mice both following saline and PACAP.

DISCUSSION

Our experimental data with behavioral, functional blood perfusion scanning and morphological techniques using gene-deleted mice confirm that PACAP plays an important excitatory role in the activation of the trigeminovascular system directly at the levels of the meningeal vessels, as well as in the trigeminal ganglia and the nucleus caudalis. A potential role has already been proposed for PACAP in the trigeminovascular system based on its immunohistochemical localization in the "migraine generator" region of the human brainstem, in the trigeminal nucleus caudalis and C1-C2 segments of the cervical spinal cord, as well as in dural mast cells. The significance of these immunolocalization results was strengthened by recent data demonstrating that PACAP infusion triggered strong headache in migraine patients and the presence of PACAP and PAC1 receptors in the smooth muscle of the middle meningeal artery.

NTG is a nitric oxide donor which leads to a direct stimulation of the trigeminovascular system at the levels of the vessels, sensory nerve terminals, as well as the TRG and the TNC leading to peripheral and central sensitization.

The light-dark box, which basically serves as a tool to determine anxiety in animals, was modified for examining light-aversive behavior. We found that in a similar system NTG induced remarkable light-aversive behavior in wildtype mice, which was significantly reduced in the PACAP deficient group.

Despite the remarkable meningeal vasodilation in response to NTG in wildtype mice, no microcirculation-increase occured in the first 2-hour period, and only a minor elevation was found in the later phase in the PACAP-deficient group. In the wildtype group c-Fos expression in the TNC increased earlier and at a greater extent than in the TRG supporting the theory that central sensitization is a crucial and initial process in NTG induced trigeminovascular activation. Significant elevation of c-Fos immunoreactivity was also observed in the TRG 4 hours after NTG injection in the PACAP$^{+/+}$ group. In contrast, c-Fos expression was not altered at either timepoints in PACAP knockout mice showing a complete lack of both central and peripheral sensitization. Therefore, PACAP proved to be a key mediator in these neuronal activation processes involved in migraine. Hyper-excitability of the trigeminal neurons both at the levels of the ganglia and the nucleus caudalis leading to an abnormal trigeminal sensory processing is a predominant pathological mechanism in migraine besides the increased meningeal vasodilation and neurogenic inflammation with plasma protein extravasation and the release of pro-inflammatory mediators from mast cells. The mechanisms by which PACAP increases the activation of the trigeminovascular system is

well-explained by the Gs and Gq protein-coupled signal transduction mechanisms related to PAC1 and VPAC receptors. The increased intracellular cAMP level leads to vasodilation, the elevation of cAMP, as well as Ca^{2+} levels induce neuronal stimulation and mast cell degranulation. PACAP and CGRP are partially co-localized in the trigeminovascular system both centrally and in the peripheral sensory nerve terminals innervating the dura, therefore, it is possible, that their excitatory actions are somehow inter-related. Selective, centrally acting receptor antagonists would be needed to elucidate these potential interactions. We found clear and similar expression of the PAC1 receptor in the trigeminal ganglia and nucleus caudalis neurons of both $PACAP^{+/+}$ and $PACAP^{-/-}$ mice pointing out that the lack of the peptide does not modulate the presence and the amount of its specific receptor in these migraine-related brain regions similarly to what was earlier found by others in other tissues. Furthermore, despite the great functional and c-Fos differences observed in PACAP-deficient mice, the PAC1 receptor expression does not change within 4 hours after NTG stimulation in either group. Although the involvement of VPAC receptors in the observed effects of PACAP in the trigeminovascular system cannot be excluded, we focused on PAC1 receptors, since a) it is its specific receptor to which PACAP has higher affinity compared to VPAC1 and VPAC2 receptors, b) nociceptive responses are markedly reduced in mice lacking the PAC1 receptor, and c) PACAP, but not VIP, has been shown to induce migraine-like headache in humans (Schytz et al. 2009). Therefore, the PAC1 receptor has been suggested to be a potential novel target for migraine.

The mean blood pressure of urethane-anaesthesized animals decreased by a similar extent (30-35%) in both wildtype and PACAP-deficient mice after this applied dose of NTG, therefore, blood pressure alterations cannot serve as an explanation for the distinct microcirculatory responses. Furthermore, despite the moderate hypotensive effect of i.p. injected PACAP, its meningeal vasodilator action was clear.

PACAP-38 enters the blood-brain barrier only at a very low ratio, less than 0.1%. Therefore, the effects we observed after i.p. injection originated from the meningeal vessels and sensory nerve terminals leading to a consequent secondary activation of migraine related brain regions. I.p. injected PACAP induced light-aversive behavior and meningeal vasodilatation similarly to NTG in wildtype, but not in PACAP-deficient mice. It significantly increased neural activation 4 hours later shown by the elevated number of c-Fos expressing cells in the TRG of both groups, but only in the TNC of the wildtype mice. The lack of vasodilation- and photophobia-inducing actions of PACAP in the PACAP-deficient group suggests that the exogenously administered peptide stimulates the peripheral terminals of PACAP-ergic trigeminal sensory nerves in the meningeal region. This results in a similar activation of the cell bodies of these pseudounipolar neurons in the TRG in both groups, but in the absence of endogenous PACAP the TNC cannot be activated. PACAP released from the central terminals of the primary sensory trigeminal neurons projecting to the TNC seems to be crucial in the central sensitization mechanisms. In contrast to what we saw for i.p. PACAP-38, i.c.v. injected PACAP-38 did not elevate the number of c-Fos-positive neurons in
either region of either group. This can be due to kinetic reasons, such as rapid elimination in the cerebrospinal fluid or bad penetration properties. The interesting observations, however, were a) the remarkably increased c-Fos expression in both regions compared to the i.p. injections in wildtypes even after saline administration, and b) the significantly reduced c-Fos immunopositivity in the TRG of $PACAP^{-/-}$ mice both following saline and PACAP. The first finding is explained by the operative procedure (drilling the skull and inserting the cannula, which is in fact a painful stimulus even under deep anaesthesia), the second observation is supported by our previous results showing a significantly decreased nociception and c-Fos expression in the pain-related brain structures of PACAP-deficient mice in a variety of pain models.

The present results are in agreement with our recently published data that remarkably reduced nocifensive behaviors originating both from exteroceptive and interoceptive areas were observed in PACAP gene-deleted animals in acute somatic and visceral chemonociception, inflammatory nociception and hyperalgesia models, as well as in the chronic traumatic mononeuropathy model.
PACAP-deficient mice display several morphological changes in brain development, such as altered cerebellar growth and abnormal axonal arborization. Furthermore, a variety of behavioral alterations have also been observed in mice lacking PACAP: they show less anxiety and explosive jumping behavior, decreased depression-like behaviors, as well as altered morphine-induced rewarding actions.
We show here that PACAP is an important mediator of trigeminovascular activation, thus, identification of its targets might be important for drug developmental purposes. Nevertheless, at present small molecule receptor type-selective antagonists, which can get into the brain, are not available. Synthesis of such compounds and their preclinical testing represent great challenges in this field.

SUMMARY OF THE NEW RESULTS, CONCLUSIONS

Our research group investigates the effects of neuropeptides released from the capsaicin-sensitive sensory nerve terminals. In my thesis I focused on the role of somatostatin and PACAP in inflammatory and nociceptive processes.
The most important results demonstrated in my thesis are the followings:

1) The sst_4somatostatin receptor plays an important role in acute and chronic inflammatory processes. In the absence of sst_4 significantly greater paw oedema developed in case of acute inflammation, and significantly increased mechanical hyperalgesia was observed both in the acute and chronic inflammation models, particularly in the early phase. In the chronic arthritis model the lack of sst_4 resulted in a remarkably less spontaneous weight bearing on the affected limb and significantly higher concentrations of IFNγ and TNFα in th ejoint homogenates. The synthetic, selective sst_4 agonist J-2156 effectively diminished acute inflammatory mechanical hyperalgesia. On the basis of these results the sst_4 receptor might be an interesting anti-inflammatory and analgesic target for drug development.

2) Somatostain and cortistatin possess similar agonistic behavior and binding profile on sst_1 and sst_4 receptors. Both peptides effectively diminish the release of the pro-inflammatory sensory neuropeptide CGRP from peripheral sensory nerve terminals, inhibit acute plasma protein extravasation in the paw skin, decrease thermal and mechanical hyperalgesia. CST and SST have similar inhibitory actions on neurogenic inflammation and hyperalgesia, but CST exerts significantly greater effect on inflammatory cytokine production.

3) PACAP plays an important role in trigeminovascular activation. Genetic deletion of PACAP in mice evoke significantly decreased nitroglycerol-induced photophobia and meningeal vasodilatation, as well as lower c-Fos expression in the TNC and TRG. PACAP released from the central terminals of primeyr trigeminal sensory neurones is a key mediator in the mechanism of central sensitization. Identification of its target molecule might open new perspectives in anti-migraine therapy.

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LIST OF PUBLICATIONS

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Helyes Z, Pintér E, Sándor K, Elekes K, Bánvölgyi A, Keszthelyi D, Szoke E, Tóth DM, Sándor Z, Kereskai L, Pozsgai G, Allen JP, Emson PC, **Markovics A**, Szolcsányi J. (2009) Impaired defense mechanism against inflammation, hyperalgesia, and airway hyperreactivity in somatostatin 4 receptor gene-deleted mice. *Proc Natl Acad Sci U S A*. 106(31):13088-93. (IF: 9,432)

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Markovics Adrienn; A szomatosztatin sst_4 receptor gyulladásgátló és fájdalomcsillapító szerepe akut és krónikus gyulladásmodellekben, egérben; *Biológus Doktoranduszok Konferenciája*, Pécs, 2009 (előadás)

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