INVESTIGATION OF THE ACTIVATION MECHANISMS OF CAPSAICIN-SENSITIVE SENSORY NERVE TERMINALS AND MEASURMENT OF THE RELEASED NEUROPEPTIDES

PhD THESIS

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INTRODUCTION:

I. 1. HISTORY AND PHARMACOLOGICAL IMPORTANCE OF CAPSAICIN

Capsaicin is the pungent principle of red pepper (*Capsicum annuum* and *Capsicum frutescens*), which is chemically an alkaloid: 8-methyl-N-vanillyl-6-nonenamide. Capsaicin acts through receptorial mechanisms. The gene of the capsaicin receptor was identified and cloned in 1997, and the name Vanilloid 1 Receptor (VR1) was given. Later the international nomenclature based on the structure of receptors altered this denomination, and this ligand-gated cation channel was classified as a member of the Transient Receptor Potential (TRP) family, it got the new name of Transient Receptor Potential Vanilloid 1 (TRPV1).

TRPV1 is a non-selective cation channel with polymodal sensor function, which can be activated by a variety of exogenous irritants, as well as endogenous physical or chemical stimuli. Besides capsaicin, there are several other substances of plant origin with vanilloid structure, which cause receptor stimulation, e.g. resiniferatoxin found in a Moroccan euphorbia (*Euphorbia resinifera*), piperin of the black pepper (*Piper nigrum*), zigeron extracted from ginger (*Zingiber officinale*) or eugenol being one of the substance of clove (*Syzygium aromaticum*). Regarding its endogenous ligands, the most important activators are noxious temperature above 43 °C, lipoxygenase products, protons (below pH 6), the endocannabiod N-arachidonoylethanolamide (AEA) called anandamide, 12-hydroperoxy-eicosatetraenoic acid (12-HPETE), N-Arachidonoyl dopamine *(*NADA).

Nociception and pain sensation develop by TRPV1 receptor activation and consequent action potential generation. Meanwhile, sensory neuropeptides are released from the nerve terminals into the innervated area, some peptides like somatostatin and pituitary adenylate-cyclase activating polypeptide, are even able to get into the systemic circulation. Continuous or repeated activation of the nerve endings results in the loss of their function (desensitization): they can not be activated by chemical stimuli, but their responsiveness to some physical stimuli remains unchanged. TRPV1 is present in a large proportion of dorsal root and trigeminal ganglia, particularly in small- and medium diameter sensory neurons, therefore, on thinly myelinated A-δ and unmyelinated C fibers.

I. 2. THE SRUCTURE AND FUNCTION OF LIPID RAFTS

It was believed on the basis of the fluid-mosaic model of Singer and Nicolson that phospholipids and proteins are randomly located in the plasma membrane. According to a new theory there are micro domains in the plasma membrane, among which lipid rafts have particular importance in regulation the activation of ion channels and receptors. The name „*lipid rafts"* comes from the fact that their components are conglomerated and able to move and swim in the plasma membrane. Lipid rafts are micro domains containing different sphyngolipids, cholesterol and proteins located among non-raft regions.

The ration of the raft components differ in different cell types, but all of them are rich in cholesterol and sphyngolipids. Proteins can be attached to the lipid rafts as transmembrane proteins, glycosylphosphatidylinositol (GPI)-coupled proteins or intracellular polypeptides like tyrosin kinases. Several proteins function as receptors or intacellular signalling mediators. Lipid rafts are in connection with the cytosceleton throughout the transmembrane proteins.

It was observed that lipid rafts are insoluble in the detergent Triton X-100 at 4 °C, in which they form glycolipid-enriched complexes. Because of their high glycolipid content, lipid rafts were named as detergent-insoluble, glycoprotein-enriched complexes (DIGs). Cholesterol is responsible for their insolubility, when cholesterol is removed, lipid rafts can be removed from the non-raft regions of the plasma membrane with Triton X-100. Therefore, with this method proteins and lipids associated with lipid rafts can be identified with different protein and lipid probes. In a variety of physiological processes lipid rafts form complexes and initiate intracellular signal tranduction pathways. Some data showed that lipid rafts play a role in regulating the function of TRP receptors forming signalling complexes with them. Besides these processes, they are involved in activation of several ligand-dependent ion channels such as the nicotinic acetylcholine receptor, the 2-amino-3-methyl-(3-hydroxy-5-methyl-isoxazole-4-il)-propionic acid (AMPA)-type glutamate receptor and the γ-aminobutyric acid (GABA) receptor. Furthermore, some G protein-coupled receptors, such as the cannabinoid 1 (CB1) recetor, have been shown to be tightly connected with lipid rafts.

Lipid rafts are directly involved in the regulation of certain cell functions. They play a role in endocytosis through the clathrin proteins found in lipid rafts. Most of the protein complexes in the lipid rafts funcion as receptors. Appropriate integrity of the rafts is needed for the adequate function of these molecules. The aim of our study was to examine the relationship between lipid raft integrity and TRPV1 receptor function.

I. 3. TRIPLE FUNCTION OF THE CAPSAICIN-SENSITIVE SENSORY NERVE ENDINGS

According to the classical theory of neural regulation, sensory nerves transmit sensory and painful stimuli from various regions of the body to the central nervous system. The other part of the peripheral nervous system is responsible for efferent functions triggered by the incoming stimuli, i.e. motoric or autonomic functions.

Capsaicin-sensitive sensory nerves expressing the TRPV1 receptor represent a unique and interesting third population. Besides the ***„classical" afferent function***, which means that their activation leads to pain sensation, they also exert ***local efferent functions.*** Pro-inflammatory neuropeptides (calcitonin gene-related peptide and substance P) are released from these fibres in response to stimulation leading to vasodilation, plasma protein extravasation and activation of inflammatory cells in the area of innervation. This phenomenon is collectively called neurogenic inflammation. In addition to the inflammatory neuropeptides staying locally in the innervated area, inhibitory peptides, such as somatostatin, opioid peptides and pituitary adenylate-cyclase activating polypeptide, are also released from the same nerve terminal, but they get into the systemic circulation. This is the third, ***systemic efferent function*** of sensory nerve endings. Our group has previously provided several lines of evidence for a potent endogenous anti-inflammatory and analgesic mechanism mediated by somatostatin released from capsaicin-sensitive sensory nerves. In addition to the well-described endocrine and paracrine inhibitory actions of somatostatin on hormone secretions, Professor János Szolcsányi introduced the term "sensocrine effect" for these inhibitory functions on inflammatory and nociceptive processes.

I. 4. SENSORY NEUROPEPTIDES RELEASED FORM CAPSAICIN-SENSITIVE NERVE ENDINGS

a.) One group of neuropeptides released from the capsaicin-sensitive nerve endings is the **tachykinins,** such as Substance P (SP), neurokinin A and B (NKA, NKB). Their effects are mediated by three G-protein coupled tachykinin receptors called NK_1, NK_2 and NK_3 receptors. SP induces plasma protein extravasation, as well as stimulates the proliferation, chemotaxis and cytokine production of a variety of immune cells via NK_1 receptor activation. SP is a cationic peptide, therefore it also gets into a non-receptor-mediated interaction with the membrane and induces mast cell degranulation. Sensory neuropeptide release is further increased through a positive feedback by histamin and serotonin acting at H_1 receptors and 5-

HT_3 receptors, respectively, located on the nerve endings. Neuropeptides are directly responsible for the acute phase of the inflammatory vascular processes, while the mediators released from mast cells (histamin, serotonin, prostaglandins, leucotriens) for later phase.
NKA shows the highest affinity for the NK_2 receptor, it induces smooth muscle contraction and stimulation of inflammatory cells (neutrophils, lymphocytes, mast cells) predominantly in the periphery, but also in the central nervous system. The NK_3 receptor binding NKB is mainly found in the central nervous system, and it has smaller importance in neurogenic inflammation.

b.) CGRP containing 37 aminoacids is found in two forms: αCGRP and βCGRP acting at CGRP1 and CGRP2 receptor. CGRP stimulates the activity of adenylate-cyclase through the CGRP1 receptor, which is G_s protein-coupled mechanism leading to intracellular cAMP increase. It activates protein kinase A (PKA) resulting in a consequent phosphorilation and opening of ATP-dependent K^+ channels. This process leads to the relaxation of the vascular smooth muscle and therefore, vasodilatation. CGRP potentiates SP-induced plasma protein extravasation by inhibiting the neutral endopeptidase being responsible for the degradation of SP. CGRP also functions as a complex immunomodulator: decreases the production of several pro-inflammatory cytokines and increases the release of the inhibitory interleukin-10 (IL-10) from macrophages. Meanwhile, it stimulates the accumulation of granulocytes.

c.) Endomorphins (endomorphin-1: EM-1; endomorphin-2: EM-2) are endogenous opioid peptides consisting of four aminoacids. These peptides are unique in comparison with other opioids having a characteristic atypical structure and high selectivity and affinity towards the μ-opioid receptor. In mammalian cells neither their genes nor their precursors are known, but they are synthetized *de novo* by certain stimuli. Endomorphins can be found in the peripheral and the central nervous systems, capsaicin-sensitive afferens and non-neuronal cells like immune cells. On the basis of their neuroanatomical localization they play a role in several physiological and pathophysiological processes, such as pain, stress responses, neuroendocrine functions and cognitive mechanisms. Most studies focus on the central effects of EMs in inflammation and neuropathy animal models, but some of them report on their anti-inflammatory, vasodilating effects as well. Because EMs do not pass the blood-brain barrier due to their hydrophilic structure, it is interesting to investigate the direct effects of these peptides synthesized in the periphery on the sensory nerve endings. EM-2 can be found predominantly in the spinal cord and several brain regions, while EM-1 is mainly produced in

sensory fibers and immune cells in the periphery. Therefore, a role of EM-1 can be proposed in peripheral inflammatory and nociceptive processes.

d.) Pituitary adenylate-cyclase activating polypeptide (**PACAP**) was originally isolated from ovine hypothalamic extracts. It belongs to the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family. The sequence of PACAP shares 68% identity with VIP, but its activating effect on adenylate cyclase is more thousand-fold greater than that of VIP. It is considered to be a sensory neuropeptide, since it is found in the dorsal horn of the spinal cord, dorsal root ganglia and capsaicin-sensitive sensory nerve terminals, but also in, the several central nervous system regions. PACAP regulates the release of neuropeptides and cell proliferation, causes vasodilatation and bronchodilatation, increases intestinal motility and inhibits apoptosis. Furthermore, it influences several physiological functions like feeding, reproduction, thermal regulation, chatecholamine synthesis and motor activities. PACAP plays a role in the development of the brain and several peripheral organs.

e.) Somatostatin (SOM) is a cyclic peptide containing 14 or 28 amino acids. Besides the capsaicin sensitive sensory nerve endings, it is widely expressed in the central and peripheral nervous sytems, neuroendocrin cells of the gastrointestinal tract, pancreas, kidney, adrenal glands, thyroid gland, inflammatory cells and genital organs. SOM inhibits hormone secretion (e.g. growth hormone, glucagon, insulin, gastrin, secretin, cholecystokinin, pancreatic polypeptide, prolactin, thyroid-stimulating hormone), gastrointestinal motility and the production of digestive enzymes. Furthermore, it inhibits the proliferation of tumor cells and has a potent immunomodulating effect. SOM acts as a neuromodulator in the central nervous system, it inhibits the release of other neurotransmitters (glutamate, serotonin, acethylcholin). It influences the locomotor activity and cognitive functions, moreover, its importance is proved in psychiatric and neurological disorders. SOM is synthetised and stored primarly in capsaicin-sensitive, TRPV1 receptor-expressing sensory neurons in the peripheral nervous system. Our group has previously proved that SOM is released from these nerve endings, gets into the circulation and exerts systemic anti-inflammatory and anti-nociceptive effects. SOM acts through five receptors (sst_{1-5}), which can be divided in two goups on the basis of binding abilities of SOM analogues. Sst_2, sst_3 and sst_5 belong to the SRIF1 group, which binds octapeptide analogues (e.g. octreotide) with high affinity, while sst_1 and sst_4 are categorized into SRIF2 which has small binding affinity to octapeptide analogues. Endocrine effects are mediated by SRIF1 receptors. In contrast, our results showed that the sst_1 and sst_4 receptors are responsible for the anti-inflammatory and anti-nociceptive effects.

II. AIMS

1. Our first aim was to investigate how cholesterol depletion of **lipid rafts** in membrane of capsaicin-sensitive sensory nerve endings influence the function of the TRPV1 ion channels. We measured the amount of the sensory neuropeptides released from the nerve terminals upon activation in an *in vitro* experimental system.

2. There were only few data available about the anti-inflammatory effect of **endomorphin-1**, the endogenous opioid peptide localized in capsaicin-sensitive nerve endings. Therefore, we investigated its actions on the prejunctional and postjunctional neurogenic (sensory nerve endings, vessels) and non-neurogenic inflammatory components (granulocytes, macrophages) both *in vitro* and *in vivo*.

3. PACAP-38 is also present in capsaicin-sensitive sensory neurons. The **PACAP6-38** fragment was used as an effictive PAC1/VPAC2 receptor antagonist in different experimental models by other groups. As a continuation of our previous investigations we examined the effects of PACAP6-38 on sensory neuropeptide release from peripheral afferents in our *in vitro* model.

4. Futhermore, our aim was to detect and measure **PACAP-38** in the plasma and milk of humans and several ruminant animals. We also investigated its alterations during pregnancy, delivery and the lactation period.

III. MODELS AND METHODS

III. 1. IN VITRO MODEL: MEASUREMENT OF NEUROPEPTIDE RELEASE FROM SENSORY NERVE ENDINGS OF ISOLATED RAT TRACHEAE

The trachea is a good model organ to investigate the activation of capsaicin-sensitive sensory nerve terminals, because it is densely innervated by peptidergic fibres, the endings are close to the surface and they can be easily stimulated. Neuropeptide release can be measured as their response. Isolated rat tracheae (2-2 organ/organ bath in 1,8 ml) were perfused with pH-(7.2) controled, oxygenized Krebs solution for 60 min (equilibration period) at 37 °C temperature. For examining the lipid rafts tracheae were incubated in Krebs solution for 30 min than in methyl-cyclodextrin (MCD) solution for another 30 min. After discontinuation of the flow, the solution was changed for 8 min to collect three fractions. The amount of the neuropeptides measured in the first period demonstrate their *pre-stimulated*, basal release. Electircal field stimulation (40 V, 0.1 ms, 10 Hz, 120 sec, 1200 pulses) or chemical stimulation (10^{-6} M capsaicin or 10 nM RTX) was performed to elicit neurotransmitter release in the second 8-min period (*stimulated* fraction). In the third 8-min period there was no stimulation (*post-stimulated* fraction), in this fraction the amount of the peptides represent the post-effect of the stimulation. The examined substances (EM-1, naloxone, PACAP1-38, PACAP6-38) were added into the incubation medium at the beginnig of each fraction. SP, CGRP, and SOM concentrations were determined from samples of the organ fluid by radioimmunoassay (RIA) methods. Peptide release was expressed as fmol per mg wet tissue weight.

III. 2. ANALYTIC STUDIES

Preparation of human samples (plasma, breast-milk) for RIA measurments

The control group composed of healthy volunteers (n=19) of both sexes (age between 20 and 40). In one experimental group healthy pregnant volunteers (n=30) between the age of 20-35 years were choosen, blood samples were taken at the end of each trimester and three days after delivery. Blood samples were also taken from the umbilical vein and artery, as well as from the superficial temporal vein of the term healthy newborns on the third day of life (n=10). In the other experimental group healthy lactating women (n=31) between the age of 20-35 years were selected, who had 1-6 month-old babies. ***Blood*** was collected into ice-cold glass tubes containing 3.7 mg EDTA and 240 U aprotinin (peptidase inhibitor). Samples were

centrifuged (1000 rpm for 10 min, and 4000 rpm for 10 min at 4 °C), the plasma fractions were stored at -80 °C. PACAP-like immunoreactivity (PACAP-LI) was determined by RIA. The peptide was extracted from 3 ml plasma by addition of a double volume of absolute alcohol and 20 µl 96% acetic acid. After precipitation and second centrifugation (3000 rpm for 20 min at 4 °C) the samples were dried under nitrogen flow and resuspended in 300 µl assay buffer to achive a concentration 10-fold higher for the RIA. ***Breast-milk*** was also collected into ice-cold tubes. For RIA analysis of milk, 10 µl 96 % acetic acid was added to 1 ml sample and incubated at 40 °C water bath for 5 min to precipitate the protein content. Centrifugation was performed at 4000 rpm for 10 min at 4 °C to obtain a solid fat fractions on the top of the samples. The whey localized between the precipitated protein and fat fractions was then removed, centrifuged (10000 rpm for 10 min at 4 °C) and used for RIA analysis.

Preparation of human samples (plasma, breast-milk) for mass spectometry

For mass spectrometry, 1 ml human ***plasma*** was filtered on a centrifugal membrane filter device. The ultrafiltration devices were centrifuged in a swinging bucket rotor for 30 min at 3000 **g**. The filtrate was liophylized and redissolved in 200 µl of 0.1 % trifluoro-acetic acid (TFA). The solution was desalted and cleaned using 0.1 % TFA and acetonitrile/0.1 % TFA (2/98, v/v) solutions with $ZipTip_{18}$ pipette tips. After elution by acetonitril/0,1% TFA (50/50, v/v) solution PACAP could be detected directly by mass spectrometry. The lipid fraction of the ***milk*** sample was preciptated by method above and the liquid phase of the mixture was loded to MALDI target plate.

Preparation of the samples of ruminant animals (plasma, milk and udder biopsy) for RIA measurments

Samples were collected from adult lactating 4-5-year-old Holstein-Friesian cows, Merino sheep and Hungarian Milk Brown goats (n= 10 of each species). Blood, milk and biopsy samples were collected in the morning, between 8-10 h at each time. ***Blood*** (10 ml per animal) was taken into ice-cold glass tubes containing EDTA (18 mg) and aprotinin (1200 U). Samples were prepared as described for the human studies. ***Milk*** was obtained during the morning milking from the same animals. Samples were preciptated as written above for RIA analysis. Blood and milk samples were collected on postpartum days 7, 30 and 90. ***Udder biops***ies were taken from lactating Merino ewes on postpartum days 7 and 30. Before sampling animals were fixed, the udders were cleaned and anesthetized locally by hipodermic

lidocaine injection. One part of tissue samples from each animal were immediately placed into liquid nitrogen, transferred to laboratory, and stored at -80 °C. Other part of samples was fixed in 4% (v/v) formaline in PBS.

PAC1 receptor-immunostaining

The tissues were immediately dissected in ice-cold PBS and fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. Then they were washed and cryoprotected in 10% sucrose for 1 h, 20% sucrose in PBS overnight at 4 °C. The samples were embedded in tissue freezing medium cut in a cryostat (10 μm radial sections), mounted on chrome-alum-gelatin coated slides and stored at -20°C until use. Tissue sections were rinsed in PBS, permeabilized by incubation for 5 min in 0.1 % Triton X-100 in PBS and incubated with 0.1 % bovine serum albumin, 1% normal goat serum and 0.1% Na-azide in PBS for 1 h to minimize nonspecific labelling. Sections were incubated with anti-PAC1 receptor antibody raised in rabbit for overnight at room temperature. After several washes in PBS, sections were incubated for 2 h at 37 °C in the dark with the corresponding Alexa Fluor „568" secondary antibody also raised in rabbit. Sections were then washed in PBS and were coversliped using Fluoromount-G. For control experiments, primary antisera were omitted and after the protocol, specific cellular staining was not found. Digital photographs were taken with a Nikon Eclipse 80i microscope equipped with a cooled CCD camera. Images were taken with the Spot software package. Images were adjusted for contrast only, aligned, arranged and labeled using the functions of the above program.

III. 3. IN VIVO EXPERIMENTS

Examination of mustard oil-induced acute neurogenic inflammation in the skin of the rat hind paw

Both hindlegs of the anesthetized rats were acutely denervated (the *sciatic* and the *saphenous* nerves were cut 30 min before the induction of inflammation) to avoid central reflexes. Acute neurogenic inflammation in the dorsal skin of the hind paw was evoked by topical application of 1% mustard oil dissolved in paraffin oil. Extravastion of plasma albumin was measured by the Evans Blue leakage method. Evans Blue (1%; 0.4 ml/100 g) 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 hind paws was removed and the extravaseted dye was extracted with formamide for 72 h at room temperature for photometric

determination at 620 nm. The ammount of the accumulated Evans Blue, which quantitatively correlates with the intensity of plasma extravasation, was expressed as μg dye/g wet tissue. EM-1 was administered i.p. 10 min before the induction of the inflammation. In a separate group, rats were pretreated with the μ-opioid receptor antagonist naloxone 15 min prior to EM-1 injection. For comparison, the solvent of EM-1 (saline) was administered after the naloxone treatment in another group of animals. To investigate whether EM-1 induces tolerance in this model, it was injected 3 times a day for 10 days and the experiment was performed afterwards.

Measurment of mustard oil-induced neurogenic edema formation of the mouse ear

Balb/c mice were anesthetized and 1% mustard oil was smeared on both sides of the ears. Ear diameter was measured with an engineers' micrometer before the treatment and four times during the 3 h-examination period. Edema was expressed in % compared with the initial control values. EM-1 was administered 15 min before mustard oil smearing. Animals of the control group were treated with the same volume of the solvent of EM-1 (saline). In a separate group of mice naloxone pretreatment was performed prior to EM-1 (8-10 mice per group).

Examination of mustard oil-induced non-neurogenic inflammation in the mouse ear

Balb/c mice were anesthesized and 1% mustard oil was smeared on both sides of the ears. To induce granulocyte accumulation this treatment was repeated every h during the 6 h-examination period. EM-1 was administered 10 min before each mustard oil smearing. Animals of the control group were treated with the solvent of EM-1 (saline). In separate groups paraffin oil (the solvent of mustard oil) was applied for comparison. At the end of the study, the right ear of each mouse was fixed in 4% buffered formalin for histological examination. Cross-sections were stained with chloroacetate esterase. The left ear samples previously frozen at -80°C were thawed and chopped into small pieces then homogenized in 50 mM potassium-phosphate buffer containing 0.5% hexadecyl trimethyl ammonium bromide (pH 6). After centrifugation (10000xg for 10 min at 4 °C), the supernatant was removed. Neutrophil accumulation was assessed by comparing MPO enzyme activity of the samples to a human standard MPO preparation. The optical density at 620 nm was measured with a micoplate reader and plotted. A calibration curve was then produced and used to define the MPO activity of the samples.

IV. RESULTS

IV. 1. LIPID RAFTS: EFFECT OF MCD ON THE RELEASE OF SENSORY NEUROPEPTIDES FROM SENSORY NERVE TERMINALS OF THE ISOLATED RAT TRACHEA

The total CGRP release induced by capsaicin was calculated by subtracting the peptide outflow in the prestimulated, basal fraction from the amount of the stimulated and post stimulated fractions, and adding the two values. In the control group 13.83 fmol/mg CGRP was released in response to 1 μM capsaicin, which was decreased significantly to 40% in the presence of 100 mM MCD. MCD did not have remarkable inhibitory effect in either smaller or higher concentrations, concentration-response correlation was not found. CGRP concentration in the incubation medium was decreased to 67% by 10 μM and to 74% by 1 mM and 10 mM, but these were not statistically significant compared to the control. The total CGRP release induced by 1 nM RTX was 11.58 fmol/mg in the control group. 100 μM MCD decreased this RTX-evoked release to 29%, but had no effect in smaller or higher concentrations similarly to capsaicin stimulation. However, the duration of the RTX effect was different compared to capsaicin: CGRP concentration did not decrease in the post-stimulated, third 8-minute fraction compared to the second fraction. The highest CGRP release was measured in the post-stimulated, third fraction.

IV. 2. EFFECT OF EM-1 ON THE RELEASE OF SENSORY NEUROPEPTIDES IN VITRO AND ACUTE INFLAMMATORY REACTIONS IN VIVO

Effect of EM-1 on electrically-evoked release of SP and CGRP

EM-1 inhibited the release of both pro-inflammatory sensory neuropeptides in a concentration-dependent manner, but it did not influence basal, non-stimulated peptide release. When analyzing the experimental data as sigmoidal concentration-response curves via a non-linear least square curve fitting procedure, maximal effects of 80.4% inhibition was obtained for SP and 85.2% for CGRP. The EC_{50} values were 39.48 nM and 10.83 nM for SP and CGRP, respectively. Co-administration of naloxone with EM-1 prevented its inhibitory action on the release of both SP and CGRP, but naloxone alone did not alter either the basal outflow or the stimulation-induced release of these neuropeptides.

Effect of EM-1 on acute neurogenic inflammation in rats

EM-1 exerted dose-dependent inhibition on 1% mustard oil-induced cutaneous plasma protein extravasation. Naloxone-pretreatment abolished the inhibitory effect of 100 μg/kg dose EM-1, but by itself it did not alter the inflammation. Analysis of these data as a sigmiodal dose-response curve via non-linear least square curve fitting procedure showed that the ED_{50} value is 1.13 μg/kg and the maximal inhibition is 58.4%. Repeated i.p. injections of the 10 μg/kg dose three times per day for 10 days did not induce desensitization in this model.

Effect of EM-1 on acute neurogenic edema of the mouse ear

EM-1 dose-dependently diminished mustard oil-induced ear swelling like in the Evans Blue accumulation rat model. The inhibitory effects of the two higher doses were significant after 1 h, wheras only the 100 μg/kg dose reached the level of statistical significance at all time points of measurment. Pretreatment with naloxone abolished the anti-edema action of 100 μg/kg EM-1, while naloxone alone did not influence ear swelling. The maximal inhibitory effect amounted to about 60% and the ED_{50} value was 1.02 μg/kg, which are very similar to that observed in the rat experiments.

Lack of EM-1 effect on mustard oil-induced late inflammatory actions

On the histological slides it was seen that repeated topical administration of mustard oil every hour throughout the 6-h-experimental period resulted in about 20-30% increase of the ear thickness and marked leukocyte accumulation as also demonstrated by our previous studies. EM-1 treatment, however, did not influence either swelling at this late time point or granulocyte accumulation.

IV. 3. AGONISTIC BEHAVIOR OF PACAP6-38 ON SENSORY NERVE TERMINALS

Capsaicin induced a 2.5-, 11-, and 3-fold elevation of SP, CGRP and SOM release, respectively. Similarly, EFS evoked a 3, 3.5- and 2.5-fold increase in the outflow of these peptides. PACAP1-38 significantly inhibited both capsaicin-induced and EFS-evoked release of these neuropeptides. Similarly, the PACAP6-38 fragment in 2000 nM dose by itself also induced significant, but smaller inhibitory actions on the release of SP and CGRP in comparison to the same concentration of PACAP1-38. In case of capsaicin-induced SOM release, the effect was similar, but its EFS-evoked outflow was decreased even in a greater

extent by PACAP6-38 than PACAP1-38. However, neither PACAP1-38 nor PACAP6-38 influenced basal, non-stimulated peptide release. When administering PACAP1-38 and PACAP6-38 together into the incubation medium, the PACAP1-38-induced inhibition was not altered or was even enhanced.

IV. 4. MEASURMENT OF PACAP-38 IN THE PLASMA AND MILK OF DIFFERENT SPECIES

Measurment of PACAP-38 in human blood and milk with Mass Spectrometry

Human serum and milk samples were measured with mass spectrometry together with PACAP-38 standard. The protonated quasimolecular ion of PACAP was successfully detected in standard, serum and milk samples as well. The post source decay MALDI TOF/TOF fragmentation of PACAP-38 standard yielded mainly y fragment ions of the PACAP-38 parent ion with aminoacid sequence. Measurements were repeated with serum and milk samples again. Similarly to standard, the peak characteristic for PACAP-38 could be detected in serum and milk samples as well, which provided evidence for the presence of PACAP-38 in human blood and milk.

Presence of PACAP-38-LI in human plasma and milk with RIA

PACAP-38 could be accurately and reliably measured in human plasma with relatively small individual differences among healthy volunteers (both sexes, age between 20 and 40 years). Age, gender or the hormonal cycle in females had no significant influence on plasma PACAP-38. However, during lactation, the plasma concentration of this peptide moderately, but significantly increased compared to the plasma level of healthy volunteers.

This RIA technique was applied to measurment of PACAP-38-LI in milk samples as well. The concentration of this peptide was 5-20-fold higher in the milk whey than in respective plasma samples. From the result of measurments among pregnant women it can be seen, that in the 2nd and 3rd trimester of the pregnancy, a moderate, but significant elevation is observed compared to the earlier gestational periods and non-pregnant condition. In contrast, during delivery, a significant, about 70% decrease was measured. Levels of PACAP in the maternal plasma returned to the original values 3 days after childbirth.

In newborn babies, PACAP-38-LI in the peripheral blood was in a similar concentration range as in healthy adults. In contrast, blood taken immediately after birth from the umbilical

arteries and veins contained significantly smaller concentration of PACAP-38. The levels were lower in the umbilical venous than in the arterial blood.

Measurment of PACAP-38 in the plasma and milk whey of ruminant animals with RIA

Similarly to the result of human samples, the concentration of PACAP-38 in the milk whey of sheep and goat was almost 10 times higher than in the plasma of respective animals. The levels did not show significant changes whithin the examined 3-month-period of lactation after birth. The presence of PACAP-38-LI was also demonstrated in the cow milk, with a serum/milk ratio similar to the other two ruminant animal species.

PACAP-38-LI in the homogenates of sheep udder biopsies

Samples were taken from sheep udder and the concentration of PACAP-38 was measured in the homogenated tissues with RIA technique. Seven days after delivery 21.07±3.39 fmol/mg PACAP-38-LI was measured in the homogenates of the mammary gland biopsies. This value decreased to 12.92±4.07 at the 30-day postpartum time-point, but this change did not prove to be statistically significant.

Immunolocalization of the PAC1 receptor in the sheep mammary gland

Immunolocalization of the PACAP-specific PAC1 receptor was clearly shown on the glandular epithelial cells of the lactating mammary gland of the sheep similarly 7 and 30 days after delivery. Negative control sections stained without the primary antibody (n=10) did not show any specific signals. The distribution of PAC1 receptor immunolabeling was structure-dependent. The glandular epithelial cells expressed remarkable and intensive immunopositivity of the PAC1 receptor, particularly in the membrane of these cells, but granular immunostaining could also be detected in the cytoplasmatic region. In contrast, the interlobular connective tissue was negative.

V. DISCUSSION

V. 1. ROLE OF LIPID RAFTS IN THE ACTIVATION OF THE TRPV1 RECEPTOR

One interesting finding in the control experiment was that the kinetics of capsaicin- and RTX-induced CGRP release from the peptidergic sensory nerve terminals was different: the maximal peptide release was evoked in the stimulated fraction in case of capsaicin, while in the post-stimulated period when RTX was applied. This observation confirm the hypothesis that capsaicin and RTX have different binding sites and activation mechanisms at the TRPV1 channel. Our results regarding the effect of cholesterol depletion in the membrane of the nerve terminal are in agreement with data obtained by us previously on the cell bodies of primary sensory neurons: damaging the lipid rafts around the TRPV1 receptor decreases its activation in case of both capsaicin and RTX. However, in both cases only the MCD 100 μM concentration was able to induce a significant inhibition on TRPV1 activation, concentration-response correlation was not found. We can conclude, that the TRPV1 receptor is located in cholesterol-rich micro domains of the plasma membrane, and their integrity is essential in its function.

V. 2. EFFECT OF EM-1 ON SENSORY NEUROPEPTIDE IN VITRO AND NEUROGENIC AND NON-NEUROGENIC INFLAMMATION IN VIVO

The present results provide the first direct evidence that the μ-opioid receptor agonist EM-1 is able to significantly and concentration-dependent manner diminish pro-inflammatory sensory neuropeptide release like SOM, sst_4 agonists and PACAP. Electrically-evoked peptide release was prevented by 1 μM tetrodotoxin as well as 25 mM lidocain, which demonstrates that activation of voltage-gated fast Na^+ channels expressed selectively on neural structures are responsible for this release process. The difference between the inhibitory effect of EM-1 on the release of SP and CGRP might be due to the fact that these peptides are not completely co-localized in the sensory nerve terminals. Several lines of evidence revealed that in sensory ganglia the largest population of neurons contain only CGRP. There are no data concerning the distribution of μ-opioid receptors on the different neural population, but based on these results it is likely that the density of these receptors might be higher on CGRP-containing terminals. Inhibitory effects of EM-1 on acute neurogenic inflammation were provided in rats

and mice in vivo as well. Repeated pretreatment with EM-1 for 10 days did not decrease the extent of observed anti-inflammatory effect, which shows that tolerance does not develop in this model. In both rats and mice the maximal anti-inflammatory effects on mustard oil-induced pure neurogenic inflammatory reactions were about 55-60% and the ED_{50} values were around 1 μg/kg i.p. dose. Edema of the mouse ear and plasma protein extravasation in the rat skin are developed by the same pathological mechanism, therefore, they can be considered as equivalent neurogenic inflammation models in two rodent species. The mouse ear swelling model is a quick, reliable, technically easy and widely used neurogenic inflammation model, which provides well-reproducible results and is accompanied by low possibilities of error. In the rat, measurment of ear swelling is not an appropriate model because of the much geater size of the ear, but determination of mustard oil-induced plasma protein extravasation in the hind paw skin by Evans Blue leakage technique is routinely used as a pure neurogenic inflammation model. Mustard oil in concentration under 5% causes pure neurogenic inflammation without the involvement of mast cells by selectively stimulating the capsaicin-sensitive sensory nerve endings. These results provided unequivocal and direct evidence that prejunctional inhibitory mechanisms play role in the anti-inflammatory effect of EM-1. The ability of naloxone to abolish the observed anti-inflammatory actions of EM-1 in all three models points out that its well-established μ-opioid receptor agonism is involved in the presently described inhibitory effects and other, non-specific mechanisms are not taken into consideration. Therefore, our results provide clear evidence that μ-opioid receptor activation on sensory nerve terminals is responsible – at least – for the ability of EM-1 to diminish neurogenic inflammation decreased SP and CGRP release.

It has been evidenced by our previous studies that mustard oil-induced granulocyte accumulation is mediated by non-neurogenic factors, sensory neuropeptides are not involved in this mechanism. Despite the ability of EM-1 to effectively inhibit the early, neurogenic component of mustard oil-induced inflammation, it was unable to alter the late, non-neurogenic phase of this inflammatory reaction. The mechanisms by which peripheral inflammatory injury enhances the effects of opioids in the periphery have been well established. Upregulation of opioid receptors on the peripheral nerve terminals of primary sensory fibers, increased efficiency of opioid receptor coupling to G proteins resulting in elevated neuronal cAMP levels and secretion of opioid peptides by immune cells at the site of injury can all occur. Under inflammatory conditions, both the expression of μ-opioid receptor in the dorsal root ganglia and their anterograde axonal transport toward the peripheral

terminals of these primary sensory neurons are enhanced. The increased number of μ-opioid receptors on the peripheral afferents could contribute to enhanced inhibitory effects of endogenous, mainly leukocyte-derived, opioid peptides and also exogenously administered ligands, which could provide promising perspectives as a novel group of drugs being able to diminish neurogenic inflammation without the development of tolerance.

V. 3. EFFECTS OF PACAP1-38 AND PACAP6-38 ON SENSORY NEUROPEPTIDE RELEASE INDUCED BY CAPSAICIN AND EFS IN ISOLATED RAT TRACHEA

Our previous data provided direct evidence that PACAP-38 decreased the release of SP, CGRP and SOM in concentration-dependent manner. The maximal inhibitory effect was 70-90% and the EC_{50} values were between 20 and 90 nM concentration, which is similar to efficacy of sst_4 agonists. This mechanism can clearly interpret data that PACAP causes bronchodilatation and inhibits the mucus production and plasmaprotein-extravasation. PACAP6-38 is used as a PAC1/VPAC2 receptor antagonist. However, the possibility of its being a partial agonist has been reported in a few studies so far, others found that both PACAP1-27 and PACAP6-27 alone could induce mild pancreatitis, and PACAP6-27 augmented the effects of PACAP1-27 in a model of acute pancreatits in rats induced by cerulein. In a recent behavioral study we have found that long-term treatment with both PACAP1-38 and PACAP6-38 in neonatal rats alters exploratory behavioral pattern in a similar way. Another study has reported that PACAP6-38 had the same effect on phagocythosis as PACAP1-38. Our experiments provided the first data on agonistic effects of PACAP6-38 caused on sensory neuropeptide release from nerve terminals of the isolated rat trachea *in vitro*. Similarly to PACAP1-38, PACAP6-38 by itself induced significant inhibitory effects on the release of sensory neuropeptides evoked by both chemical excitation and EFS of capsaicin-sensitive afferents. Capsaicin selectively activates the TRPV1 receptors on these nerve endings, while EFS with the applied parameters opens fast Na^+ channels expressed specifically on the peptidergic afferents. Therefore, we are convinced that the applied stimulation parameters are selective for capsaicin-sensitive sensory fibers of the trachea. In comparison to 2000 nM PACAP1-38, the extent of the inhibition induced by the same concentration of the fragment was smaller in most cases, which could raise the possibility of its being a partial agonist. However, administration of PACAP6-38 together with PACAP1-38 did not block or decrease its inhibitory effect on the peptide release. Furthermore, the co-administration even enhanced the inhibition compared to the effects of PACAP1-38 alone in

cases of capsaicin-induced SOM or EFS-evoked SP release. Therefore, its partial agonistic profile is not likely; it can clearly been considered as an agonist in this system. According to previous data, as well as the present results, the effects of PACAP6-38 seem to be tissue-or target-cell-dependent.

V. 4. PRESENCE OF PACAP-38-LI IN THE PLASMA AND MILK OF HUMANS AND RUMINANT ANIMALS

Our results revealed that PACAP-38 is present in human plasma and its concentration is relatively stable in 20-40 year-old healthy volunteers. It is not significantly altered by gender, age, food intake or hormonal cycle in females. The exact source of PACAP in the plasma is not known, it is most likely to be derived from neural and endocrine elements. We have recently shown the elevation of plasma PACAP-38-LI in rats by chemically stimulating sensory nerve endings throughout the body.

These data are the first which provide evidence for the presence and high concentration of PACAP-38 in the human milk; its levels are 5-20-fold greater in the milk whey than in the respective plasma samples. Moreover, our examinations showed that PACAP-38 is present in the milk of domestic animals at concentration comparable to human milk. Highest levels were found in goat and sheep milk, while cow milk contained significantly lower levels. Futhermore, similarly to human milk, concentrations of PACAP in the milk of these animals exceeded those of plasma by 5-20-fold. The finding that cow milk contained the lowest levels of PACAP is in accordance with the observation of others showing that cow milk contains lower levels of growth factors than human or goat milk.

Milk contains several gastrointestinal neuropeptides, like bombesin, peptide YY, neurotensin, gastrin, cholecystokinin and peptide histidine methionine. A supposed function of these peptides is the regulation of growth and maturation of the gastrointestinal system in neonates. Several growth factors are present in higher concentrations in the milk than in the plasma, such as gonadotropin releasing hormone, thyreotrop hormone, VIP, SOM, growth hormone releasing hormone, relaxin, IGF-1, epidermal growth factor and prostaglandins. These bioactive componds may play a role in the growth of the mammary gland and funcion of nutrient transfer and regulation of growth and differentiation of neonatal tissues.

The role of PACAP in milk can only be hypothesized at the moment. Being present at such high concentrations suggests that PACAP is required for the growth and development of the newborn. PACAP has been shown to play very important roles during the development of

various organs, especially the nervous system. PACAP functions as a neurotrophic factor from very early stages of neuronal development influencing neurogenesis, differentiation and patterning, and it is also involved in processes that continue after birth, such as astrocytogenesis, myelination, cerebellar development and neuronal migration. Multifunctional milk components protect against several pathogens and also stimulate the development of the natural defensive mechanisms. PACAP has a variety of immunomodulatory actions, both during the development of the immune system and in mature lymphocytes and macrophages. Another possible function could be the regulation of growth and function of the mammary gland itself. PACAP is well-known for its antiapoptotic actions and its involvement in regulating cell cycle. Apoptosis play a key role also in the development of the mammary gland. Influenced by growth factors, the expression of antiapoptotic molecules is high during lactation, while the involuting mammary gland undergoes massive cell loss by apoptosis.

A further important question is the oral bioavailability of PACAP derived from the milk. PACAP is cleaved by dipeptidyl peptidase IV, which is the major factor limiting the half-life of biologically active peptides. It has been shown that the half-life of PACAP and related peptides is relatively short (minutes) in body fluids. The mammary gland produces protease inhibitors that are resposible for the stability of variuos milk-borne proteins and peptides. Neonates possess lower proteolytic activity, including immaturity of the dipeptidyl peptidase IV, and have a higher permeability for macromolecules allowing significant absorption of proteins/peptides through the intestinal epithelium.

Our human study examined pregnant women is the first to show that PACAP-38-LI increases by the 2nd and 3rd trimester of the pregnancy, it decreases during delivery, and returns to normal levels 3 days after childbirth in healthy women. In newborn babies, PACAP-38-LI is similar to that measured in the adult plasma, while blood taken from the umbilical vessels contains lower levels of PACAP, with the venous blood showing significantly less PACAP than the arterial blood. Although the function of PACAP in these physiological conditions can not be speculated based on the present data, our sudy shows endogenous PACAP levels sensitively react to the hormonal changes under these conditions. Furthermore, the finding that the umbilical artery contains higher level of PACAP than the vein suggests that the fetal organs actively synthetize this peptide.

PACAP is a pleiotropic and multifunctional neuropeptide about that several lines of evidence suggest its importance in regulating female hormones also related to pregnancy, fertility, as well as uterine smooth muscle contraction and blood flow. The higher levels of PACAP-LI

found in late pregnancy (after 2nd trimester) shows that the placenta and/or other maternal organs synthesize higher levels of peptide. This is in accordance with our recent finding showing that the placental PACAP content increases during pregnancy. Based on the present results, PACAP-38 can be proposed to play a physiological role during pregnancy, but elucidating these processes and mechanisms need further investigations. We do not know the source of PACAP found in the umbilical vessels, but its higher levels measured on the arterial side indicates that this difference is due to PACAP synthesis in the fetus. The appearance of PACAP has been shown at very early stages of development, from zebrafish to mice. Deficiency of PACAP has been proven to result in several developmental alterations in mice, from pathological cerebellar development to behavioral alterations. Although further research is needed to reveal the functional significance of PACAP in human pre-natal and post-natal development, the present findings are particularly important to initiate investigations in this area.

VI. SUMMARY OF THE NEW RESULTS, CONCLUSIONS

1. We proved that TRPV1 receptors are located in cholesterol-enriched micro domains of the membrane of sensory nerve terminals, the integrity of these micro domains is essential for receptor activation and consequent neuropeptide release.
2. We showed that the endogenous opioid peptide, endomorphin-1, is able to decrease the release of sensory neuropeptides from capsaicin-sensitive afferents and therefore, acute neurogenic inflammation. However, it has no effect on the late, non-neurogenic cellular components of the inflammatory processes.
3. We provided evidence that PACAP6-38 used as a PAC1/VPAC2 antagonist in several models acts as a potent agonist similarly to PACAP1-38 on the peptidergic capsaicin-sensitive sensory nerve endings: it inhibits neuropeptide release in a concentration-dependent manner. The effects of PACAP6-38 seem to be tissue-or target-cell-dependent. Since this inhibitory action can not be explained by the known G_s and G_i-coupled singal transduction mechanisms of PACAP, a new receptor or splice variant can be proposed on the nerve endings. Examination of these mechanisms, indentification of the target molecule, and analysis of the signal transduction pathway are the aims of our future investigations.
4. We provided the first evidence that PACAP-38 is present in the plasma and milk of humans and ruminant animals. Its plasma level is altered during pregnancy and delivery: plasma PACAP-LI gradually increases during pregnancy, then decreases significantly during delivery, and returns to the original values 3 days afterwards. The finding that the umbilical artery contains higher level of PACAP than the vein suggests that the fetal organs actively synthetize this peptide.

   PACAP-38 levels were 5-20-fold higher in the milk compared to the respective plasma samples both in humans and ruminant animals. Further research is needed to reveal the functional significance of these results and to elucidate the potential role of PACAP in human pre-natal and post-natal development, and the proliferation of the mammary gland and the lactation process.

VII. PUBLICATIONS

PUBLICATIONS RELATED TO THIS THESIS

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Cummulative impact factor of other publications: 22.226
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Cummulative impact factor of all publications: 35.966
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