INTRODUCTION, BACKGROUND OF THE RESEARCH

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 transmission of nociceptive information into the central nervous system, which leads to pain sensation. Besides this process, pro-inflammatory 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. Furthermore, these sensory neuropeptides modulate inflammatory and immune cells [39,61,115,116]. These pro-inflammatory actions refer to the local efferent function of capsaicin-sensitive afferents. The neurogenic inflammatory component plays an important role in the pathological mechanism of several diseases, e.g. rheumatoid arthritis, asthma, psoriasis, ekzema, allergic contact dermatitis and inflammatory bowel diseases [60,114]. The presently available anti-inflammatory drugs are not able to inhibit the neurogenic processes of these inflammatory reactions, their pharmacotherapy is still not satisfactory [39].

The third, systemic efferent function of capsaicin-sensitive sensory nerves was discovered about 10 years ago in our research group. Several lines of evidence have been provided that besides the locally liberated pro-inflammatory neuropeptides, somatostatin is also released from the activated afferents, reaches the circulation and exerts systemic anti-inflammatory and analgesic actions [38,41,111,112]. This endogenous counter-regulatory mechanism of sensory nerve-derived somatostatin has been defined as its „sensocrine function" parallel to its well-known endocrine and paracrine actions [110].

Sensory neuropeptides

1. Pro-inflammatory and pro-nociceptive neuropeptides

One group of pro-inflammatory sensory neuropeptides released from stimulated capsaicin-sensititve sensory neurons is called tachykinins, which includes substance P, neurokinin A and neurokinin B (NKB). They exert their effects via G protein-coupled neurokinin receptors, namely NK_1, NK_2, NK_3. SP enhances vascular permeability via NK_1 receptors mainly expressed on postcapillary venules, machrophages, limphocytes, polymorphonuclear and mast cells [11,80,89], which results in plasma protein extravasation, T cell proliferation, chemotaxis, activation of mast cells or neutrophil accumulation [29]. NKA induces smooth muscle contraction, plasma protein extravasation and stimulation of inflammatory cells (neutrophils, lymphocytes, macrophages) through NK_2 receptor activation [17] in the periphery and the central nervous system. NKB binds to NK_3 receptors, which is mainly present in the central nervous system and in some peripheral organs [27,63].

The 37 amino acid-containing CGRP was discovered by Amara and co-workers [2]. It exists in two variant forms, αCGRP and βCGRP both acting on G_s protein-coupled receptors called $CGRP_1$ and $CGRP_2$ [66,73,124]. CGRP belongs to the calcitonin/CGRP peptide family, other members of which are calcitonin, islet amyloid polypeptide and adrenomedullin [85]. They posess strong vasodilator effect mainly via $CGRP_1$ receptor activation. Possible mechanism of CGRP-evoked vasodilation is activation of ATP-sensitive potassium channels by the protein kinase A, which is activated by the increased amount of intracellular cyclic AMP. This mechanism leads to relaxation of vascular smooth muscle cells [23,32,46]. Although CGRP itself does not induce directly vascular permeability, but potentiates the action of SP which makes this peptide important in the development of neurogenic inflammation [10]. Moreover, it has complex immuno-modulatory function, CGRP inhibits the secretion of pro-inflammatory cytokines (IL-1, IL-12) by macrophages, but facilitates the production of the anti-inflammatory IL-10 and enhances the granulocyte accumulation [4].

2. Anti-inflammatory and anti-nociceptive neuropeptides

Somatostatin, also known as growth hormone (GH) or somatotropine release inhibitory factor (SRIF) is a 14 or 28 amino acid-containing cyclic peptide [9]. Somatostatin is widely distributed in the central and peripheral nervous systems [79,90], the a gastrointestinal tract, the pancreas, the lung, the adrenal and tyroid glands, inflammatory cells and gonads [42,91,122]. It inhibits the secretion of several hormons, the motility of the gastrointestinal tract, gastric/bowel juice secretion and the proliferation of tumor cells. Somatostatin modulates cognitive functions, its importance is proved in several neurological and psychiatric diseases [128].

Somatostatin exerts its physiological effects via G_i protein-coupled receptors. Five human somatostatin receptors have been cloned in mice, rats and humans, which are called sst_1, sst_2, sst_3, sst_4 és sst_5 [81]. These five receptors can be divided into two different subgroups: the SRIF1 group comprises of sst_2, sst_3 and sst_5, whereas the SRIF2 group contains sst_1 and sst_4 [45,82]. The endocrine functions are mediated via receptors of the SRIF1 group [87]. The anti-nociceptive and anti-inflammatory effects are related to the other group, SRIF2, which our research group focuses on [40,82,83,110].

Pituitary adenylate cyclase activating polypeptide (PACAP) was originally isolated from ovine hypothalamus [68]. PACAP is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family and exists in two forms: the 27 amino acid-containing PACAP-27 and the 38 amino acid-containing PACAP-38, which is predominant in mammals [95,97,136]. Highest concentrations can be found in the hypothalamus and in the gonads [3, 99,100], but it is also expressed in other areas of the central nervous system, in sensory neurons of dorsal root ganglia [21,70,78], in endocrine glands [67,129], in the gastrointestinal tract [33], in the respiratory tract [69], and in the skin [75]. PACAP-38 has diverse actions in the central and peripheral nervous systems and also in peripheral tissues: it is reported to regulate cell proliferation and to promote apoptosis in tumor cells [126]. PACAP modulates the release of neurotransmitters [64], evokes vaso- or bronchodilation, enhances the gastrointestinal motility, increases hormone concentrations in the blood [31]. Two types of PACAP binding sites were evidenced in different tissues. Type I binding sites have high affinity for PACAP-38 and PACAP-27, but low affinity for VIP [28,55,106]. Type II binding sites have similar affinity for PACAP and VIP [121,127], in this group there are two subtypes based on the binding affinity to secretin. These receptors have been cloned and they are now called PAC1 and VPAC1/VPAC2, respectively [34].

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, which can be divided into two well-defined components. Its neurobiological component is nociception (perception of painful stimuli, sensory experience), which can be examined in animal experiments, while 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), thermal (heat) or chemical (capsaicin, formalin, acetic acid, etc.) stimuli. Acetic acid-, $MgSO_4$- or phenylquinone-induced writhing test in the mouse is appropriate to study visceral pain originating from interoceptive areas (internal organs, pleura, peritoneum, pericardium). Somatic pain derived from cutaneous afferents can be examined with the formalin test both in rats and mice. 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. Both mechanical and thermal allodynia as well as hyperalgesia can occur in inflammatory conditions, peripheral neuropathy of different origin (traumatic, toxic) or after neural damage on any level of the spino-thalamo-cortical system [119]. Partial tight ligation of the sciatic nerve [96], loose ligation of the whole nerve [5] or ligation of the L5 spinal nerve [50] serve as commonly used animal models for peripheral traumatic neuropathy.

Need for the development of novel analgesics and anti-inflammatory drugs

Since only nociception can be studied in internationally accepted animal models of pain with the well-defined experimental techniques, determining the effect of novel analgesic drug candidates in the pre-clinical phase of drug development is not an easy task. The treatment of neuropathic pain is still an unresolved problem, the presently available drug groups (anti-epileptics, opiates, anti-depressants, lidocaine) do not provide satisfactory relief in most cases. The number needed to treat (NNT) value which shows the number of patients required to be treated with certain drugs so that 50% pain-relief could be achieved in the first one is used for comparison of the effect of these drugs [119]. The classical non-steroidal anti-inflammatory/analgesic drugs all act by inhibiting the cyclooxygenase enzyme and decrease prostaglandin synthesis. Although they have been used in the clinical practice for centuries, they are unable to affect neuropathic pain and the neurogenic components of the inflammatory processes. Opioids, which are very effective in cancer pain, can only be considered as second or third line drugs in certain neuropathic conditions such as postherpetic or trigeminal neuralgia, diabetic polyneuropathy [119]. High dose corticosteroids inhibit neurogenic inflammation, but due to a great range of severe side effects (gastrointestinal bleeding, ulcer formation, diabetes, obesity, etc.) they cannot be administered for longer time periods. Therefore, there is a particularly great need to find new targets and develop novel anti-inflammatory and analgesic drugs with different mechanisms of action which could act directly at the level of the sensory nerve terminals. For this reason we have introduced and developed several animal models of nociception in our laboratory and I have obtained expertise in a variety of examination techniques during my PhD work.

AIMS

My PhD thesis deals with the mechanisms and receptorial background of peptide transmitters which are released from capsaicin-sensitive sensory nerves and mediate the neurohumoral „sensocrine" anti-inflammatory and anti-nociceptive actions of these fibres and also focuses on the abilities for their physical activation in animal models. This work is built around the following three topics.

I. Our aim was to investigate the role of the somatostatin receptor subtype 4 (sst_4) in different *in vivo* models of inflammation and nociception. The effect of the selective sst_4 receptor agonist J-2156 having a peptidomimetic structure was studied in acute and chronic experimental assessments both in rats and mice. Besides, on the basis of the literature I introduced an *in vitro* method in which we could routinely measure the cytokine production of isolated peritoneal macrophages in response to different stimulations and examine drug effects.

II. The presence of PACAP-38 in capsaicin-sensitive sensory neurones has been established and our previous studies have proved that it is released from their peripheral terminals in response to stimulation. However, the available contradictory data concerning its role in nociceptive processes all focused on the central nervous sytem. Therefore, in the present series of experiments the peripheral actions of PACAP-38 was investigated in a variety of nociception models.

III. With the help of a specific device producing a static magnetic field with optimized parameters we studied if this physical influence is able to induce measurable anti-nociceptive actions in different mouse models. Furthermore, the potential involvement of capsaicin-sensitive peptidergic fibres was also analyzed in the observed phenomenon.

I. ANALGESIC EFFECTS OF THE SOMATOSTATIN SST_4 RECEPTOR SELECTIVE AGONIST J-2156 IN ACUTE AND CHRONIC INFLAMMATORY AND PAIN MODELS

The therapeutic value of native somatostatin is limited by its broad range of effects and its short (3 minutes) plasma half life [122]. However, potent and stable somatostatin receptor agonists acting selectively on sst_4 receptors on nociceptive nerve terminals could be promising for analgesic drug development. 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 agonists molecule has wide range of anti-nociceptive effects, which is supported by our earlier studies [37,38,41,83]. Our present experiments were performed on a sst_4 receptor subtype selective, high affinity binding somatostatin agonist, compound J-2156, which is synthesized at Juvantia Pharma Ltd. (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 posesses nanomolar affinity to the human somatostatin receptor subtype 4 (sst_4), which is a greater response of binding affinity than the native somatostatin's, and is at least 400-fold selective for the sst_4 receptor over any of the other four human somatostatin receptor subtypes [25]. In a cyclic AMP assay J-2156, somatostatin-14 and somatostatin-28 all act as full agonists. In a G protein stimulation functional test J-2156 elicited a 2.5-fold higher response than the endogenous ligands somatostatin-14 and somatostatin-28. By these results J-2156 can be classified as a „superagonist" at human sst_4 receptor [25]. Further *in vitro* experiments provided evidence that repeated application of compound J-2156 did not cause desensitization, which enhances the therapeutic value of this molecule [24].

I.1. EXPERIMENTAL MODELS AND METHODS

Animals

Experiments were performed on Balb/c, CD1 mice, and Wistar, Lewis rats bred and kept in the Laboratory Animal Center of the University of Pécs under standard pathogen free conditions at 24-25°C and provided with standard chow and water *ad libitum*.

Investigation of formalin-induced acute somatic chemonocifensive behaviour

Formalin (50 μl 2.5%) was injected intraplantarly into one hindpaw of Balb/c mice, which induced nocifensive reactions in two phases. Phase I (0-5 min) is thought to be due to a direct chemonociceptive effect of formalin. Phase II (20-45 min) is mainly mediated by inflammatory reactions [123]. Nocifensive behaviour was quantified in seconds by the duration of paw lickings in both phases. J-2156 was administered in 3 doses (1, 10 and 100 μg/kg) intraperitoneally 20 minutes prior to formalin-injection.

Measurement of mechanonociceptive threshold of the paw in traumatic mononeuropathy

Wistar rats 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, which causes a drop in the mechanonociceptive threshold [96]. The wound was closed and the animals were allowed to recover and survive for 7 days. Mechanonociceptive threshold of the hindpaws was determined with Randall-Selitto-test (Ugo Basile Analgesimeter) before and 7 days after surgery, which is a reliable method for measuring mechanonociception in the partial sciatic nerve ligation model [83]. J-2156 was administered in 3 doses (1, 10 and 100 μg/kg i.p.) 20 minutes before measurements.

Investigation of complete Freund's adjuvant-induced chronic inflammation

Complete Freund's adjuvant (CFA: killed *Mycobacterium tuberculosis* suspended in paraffin oil, 1 mg/ml) was injected intraplantarly and into the tail's root (100-100 μl) of Lewis rats. In order to enhance

systemic effects, an additional injection was given into the tail the following day, this was the first day of the experiment. Functional measurements were performed before and for 21 days after CFA-administration. At the end of experiments tibio-tarsal joints were cut, histological preparation and morphological evaluation has been made. J-2156 was given in two different doses (1 and 10 μg/kg i.p.) three times daily throughout the whole experimental period from the start of CFA-treatment.

Measurements of mechanical touch sensitivity of the paw

Mechanonociceptive thresholds was measured by Ugo Basile Dynamic Plantar Aesthesiometer, which is a modified electronic von Frey device. The results was compared to the initial control values, consequent allodynia was expressed in percent.

Measurements of oedema formation

Paw volume was measured with Ugo Basile Plethysmometer. Oedema formation was expressed in percentage compared to the initial control volumes.

Histological preparation and evaluation

At the end of the measurements tibio-tarsal joints were cut and decalcinated, dehydrated with adequate protocol. The samples were embedded in paraffin, then sectioned with microtome to 5-7 μm pieces and stained with hematoxylin and eosin [38]. Arthritic changes of each samples were scored with formally given parameters by a pathologist without knowledge of the treatments received. We used a grading scale of 0–3 for each parameter where the biggest score means the most serious changes. Mean scores were determined from each sections of the individual animals and composite score values of different experimental groups were calculated from these mean scores [132].

Investigation of carrageenan-induced oedema formation

3% carrageenan (100 μl) was injected into the plantar surface of one hindpaw of Wistar rats, which induced a local mixed-type inflammatory response with neurogenic and non-neurogenic components [20,84]. The oedema formation achieves its maximum in the third hour [8], so we have made our measurements before and 60, 120 and 180 minutes after injection. Three doses of J-2156 (1, 10 and 100 μg/kg) or saline was administered intraperitoneally 15 minutes prior to carrageenan injection.

Investigation of mustard oil-induced acute neurogenic inflammation in the rat hindpaw skin

Both hindlegs of Wistar rats were acutely denervated, the sciatic and the saphenous nerves were cut 30 minutes before the induction of inflammation to avoid mustard oil-induced central reflexes. Acute neurogenic inflammation in the paw skin was evoked by topical application of 1% mustard oil dissolved in paraffin oil. Extravasation of plasma albumin was measured by the Evans blue leakage method. Evans blue (50 mg/kg) was injected intravenously and neurogenic inflammation was induced 10 minutes later. Rats were exsanguinated 20 minutes after mustard oil application. The skin of the hindpaws was removed, weighed and the extravasated dye was extracted with formamide. The amount of the accumulated Evans blue, which quantitatively correlates with the intensity of plasma extravasation, was quantified with photometric determination at 620 nm. The results were expressed in μg dye/g wet tissue. Doses of J-2156 (0.5-100 μg/kg) or saline was administered intraperitoneally 20 minutes before induction of the inflammation. For determine the duration of action, J-2156 (10 μg/kg) was administered intraperioneally 2 or 6 hours before mustard oil-smearing.

Investigation of mustard oil-induced acute neurogenic inflammation in the ear

Balb/c mice were anaesthesized and 1% mustard oil dissolved in paraffin oil was smeared on both sides of the ears. The diameter of the ear was measured with an engineers' micrometer before the treatment and four times during the 3 h-examination period. Oedema was expressed in percent compared to the

initial control values. J-2156 (10, 50 and 100 μg/kg i.p.) was administered 15 minutes before mustard oil-smearing. Animals of the control group were treated with the same volume of the solvent.

Examination of IL-1β production of stimulated peritoneal macrophages *in vitro*

CD1 mice were treated with lipopolysaccharide, LPS (300 μl i.p., 300 μg/ml). Four hours after the injection animals were decapitated and desanguinated. The abdomen was washed with 2.5 ml ice-cold medium and the peritoneal liquid was collected. 800 μl RPMI (with 80 μl BSA) and 100 μl peritoneal liquid was pipetted into each well of the cell culture plate. We have made two different settings for stimulating inflammatory cells: in one we pipetted 1-1 μl 1 μg/ml LPS to the medium, in the another 1-1 μl 25 ng/ml phorbol-12-myristate-13-acetate (PMA) and 1-1 μl 1 μg/ml ionomycin was pipetted into each well. After that one half of each plate we have pipetted in 100 μl saline, the other half four different concentrations (0.1, 1, 10 and 100 μg/ml) of J-2156 (100 μl) was put in to examine the changes in cytokine-concentration. The amount of IL-1β produced by the samples were measured with sandwich ELISA method and determined with spectrophotometry at 450 nm.

Statistical analysis

Statistical evaluation of the formalin test, the mustard oil-induced acute neurogenic inflammation, and the two models investigating mechanonociception was analysed with non-parametric Mann-Witney U-test. For the histological changes Kruskal-Wallis test, for the *in vitro* model Student's t-test, for the oedema formation one-way ANOVA followed by Bonferroni's modified t-test was used. In all cases comparing the different groups *p<0.05, **p<0.01 and ***p<0.001 was considered to be significant.

I.2. RESULTS

Inhibitory effect of J-2156 on formalin-induced acute nocifensive reactions in the mouse

In phase I of formalin test (0-5 minutes) J-2156 proved to be ineffective, it did not influence the total duration of paw lickings. Meanwhile, in the second, acute inflammatory phase (20-45 minutes) J-2156 showed a significant and dose-dependent anti-nociceptive action.

Anti-hyperalgesic effect of J-2156 in rat traumatic mononeuropathy model

Partial ligation of the sciatic nerve resulted in a significant decrease of the mechanonociceptive threshold, which was not altered by either the 1 μg/kg i.p. dose of J-2156 or the solvent (saline). However, 10 and 100 μg/kg i.p. J-2156 significantly, but not dose-dependently decreased mechanical hyperalgesia.

Inhibitory effect of J-2156 on CFA-induced inflammatory mechanical allodynia

In control rats CFA-injection caused a marked decrease in mechanical touch sensitivity, which was maintained throughout the whole experimental period. Both doses of J-2156 (1 and 10 μg/kg i.p.) induced a significant inhibition on mechanical allodynia with only a moderate dose-reponse relationship.

Inhibitory effect of J-2156 on CFA-induced oedema formation

In the control, saline-treated, group paw swelling gradually increased and remained unchanged for the whole 3-week period. Both doses (1 and 10 μg/kg i.p.) of J-2156 significantly decreased oedema formation at almost every measurement point. However, dose-response correlation was not found.

Inhibitory effect of J-2156 on histological changes

The composite arthritis score for the saline-treated group was 6.6±0.7. The treament with J-2156 three times a day for 21 days markedly decreased the changes, the arthritis score was 2.86±0.86 in response to the 1 μg/kg i.p. dose, and 4.5±0.56 for the 10 μg/kg i.p. dose of J-2156.

Inhibitory effect of J-2156 on carrageenin-induced oedema formation

Intraplantar injection of carrageenan induced 30, 32 and 27% paw swelling in control, saline-treated rats at 60, 120 and 180 minutes, respectively. This oedema was inhibited by pretreatment with 10 and 100 μg/kg J-2156 at each measurement points. This inhibitory effect was not dose-dependent in this model.

Inhibitory effect of J-2156 on mustard oil-induced acute neurogenic inflammation in the paw skin

Mustard oil applied to the paw skin induced a marked extravasation of plasma protein, assessed by leakage of Evans blue dye. This inflammation was inhibited non-dose-dependently by six different doses (between 0.1 and 100 μg/kg) of J-2156 injected 20 minutes before mustard oil. The significant inhibitory action of 10 μg/kg J-2156 lasted for 6 hours.

Inhibitory effect of J-2156 on mustard oil-induced acute neurogenic oedema of the mouse ear

In the control group ear thickness markedly increased within 3 hours in response to topical application of 1% mustard oil. All three doses of J-2156 pretreatment (10, 50 and 100 μg/kg i.p.) significantly diminished mustard oil-induced ear swelling after 2 and 3 hours, also. Only the effect of 50 μg/kg J-2156 reached the level of statistical significance already at 20 minutes. Dose-dependency has not been found.

Inhibitory effect of J-2156 on IL-1β release from *in vitro* stimulated peritoneal macrophages

Both LPS and PMA stimulation of isolated mouse peritoneal macrophages induced similar IL-1β production. Compared to the control, saline-treated cells, pretreatment with 1 and 10 μg/ml J-2156 caused a sigificant decrease in the release of this cytokine. On the contrary, the 0.1 μg/ml concentration was uneffective and the highest concentration (100 μg/ml) surprisingly increased the IL-1β production.

I.3. SUMMARY AND CONCLUSIONS

Our present results clearly demonstrate that the peptidomimetic compound J-2156, a highly somatostatin sst_4 receptor selective agonist, was able to significantly inhibit nocifensive reactions in a conventional acute somatic chemonociception test (formalin) in mice, as well as in two different chronic pain models (adjuvant-induced chronic inflammation and traumatic mononeuropathy) in rats, indicating its broad-spectrum anti-nociceptive and anti-hyperalgesic/anti-allodynic effects in both species. It significantly inhibited acute inflammation in the rat hindpaw skin and in the mouse ear. The anti-inflammatory effect of J-2156 has been shown in the *in vitro* cell stimulation assay, J-2156 inhibited IL-1β production of isolated peritoneal macrophages in response to both LPS and PMA stimulation.

Our earlier data showed that this agonist inhibited the release of SP and CGRP from stimulated sensory nerve terminals of the isolated rat trachea [36]. This mechanism is involved –at least partially- in the described anti-inflammatory and analgesic actions. Although only a few data are availabe on the exact localization of sst_4 receptors [82], these functional results with J-2156 show that they are likely to be localized directly on the nerve terminals, vascular endothelial cells and inflammatory cells. This latter theory is supported by our i*n vitro* data obtained on peritoneal macrophages.

Since J-2156 acts selectively on sst_4 receptor, it avoids the most common endocrine side effects of somatostatin agonists (inhibition of growth hormone, glucagon and insulin release) as these effects are mainly ascribed to SRIF1 receptor family [131]. This study provides several lines of evidence that the sst_4 receptor is an excellent target for drug development. Therefore, stable, selective sst_4 agonists can open promising perspectives for novel anti-inflammatory and analgesic drugs.

II. DIVERGENT PERIPHERAL EFFECTS OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE-38 (PACAP-38) ON NOCICEPTION IN RATS AND MICE

Pituitary adenylate cyclase activating polypeptide (PACAP-38) is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family that was originally isolated from ovine hypothalamus [68]. Highest concentrations of PACAP are found in the nervous system and endocrine organs. PACAP serves as a sensory neuropeptide based on the presence of PACAP-like immunoreactivity in the superficial dorsal spinal horn layers, cell bodies [18,19] and peripheral terminals of capsaicin-sensitive primary sensory neurons [26,139]. Furthermore, PACAP and its receptors have been also detected in the dorsal horn of the spinal cord [69,70] as well as the articular capsule [125]. On the basis of these morphological and molecular biological results, PACAP has been suggested to be involved in pain transmission, but very few functional data are available to support this theory [35,72]. All *in vivo* experiments studying its role in nociception focused on its central effects and the results are contradictory [98]. Intrathecally injected PACAP inhibited spinal nociceptive reflexes [140] and inflammation-induced nociception [77,137,139]. Administration of PACAP intracerebroventricularly also resulted in analgesia in the early phase and algesia in the late phase [98]. On the other hand, central application of PACAP dose-dependently decreased paw withdrawal latencies induced by thermal stimulation and potentiated nociceptive transmission to the spinal dorsal horn by interacting primarily with N-methyl-D-aspartate (NMDA) receptors [76]. PACAP acts via $G_{s/q}$ protein coupled receptors mainly associated with the adenylate cyclase and phospholipase C: the PAC1 receptor which specifically binds PACAP, and the VPAC1/VPAC2 receptors which have a similar binding affinity for PACAP and VIP. Both receptors have been described on neurons, smooth muscle cells and several inflammatory cells [18,104,127,142].

Our earlier data provided evidence that PACAP inhibits the release of pro-inflammatory/pro-nociceptive sensory neuropeptides: substance P (SP) and calcitonin gene-related peptide (CGRP) from peripheral terminals of capsaicin-sensitive nerves [72]. PACAP also inhibited acute neurogenic and non-neurogenic inflammatory processes in both mice and rats [38,72]. Based on these results it was tempting to assume that this peptide might be involved in peripheral mechanisms of nociception.

Since there were no data available on the peripheral actions of PACAP-38 in nociceptive processes, the present study aimed at examining its effects on acute visceral and somatic nocifensive behaviors, as well as inflammatory and neuropathic mechanical allodynia and heat injury-evoked thermal hyperalgesia after local or subcutaneous/intraperitoneal administration in different rat and mouse models.

II.1. EXPERIMENTAL MODELS AND METHODS

Animals

Experiments were performed on Wistar rats and CD1 mice bred and kept under pathogen free conditions at 24-25°C and provided with standard chow and water *ad libitum* in the Department of Pharmacology and Pharmacotherapy and Laboratory Animal Center of the University of Pécs, respectively. Wistar rats used for the electrophysiology studies were kept at the Animal House of the University of Calgary under the same conditions.

Investigation of formalin-induced acute somatic nocifensive behaviours

50 μl 2.5% formalin injected subcutaneously into the plantar surface of one hindpaw of Wistar rats induces nocifensive reactions which is different from the behaviour in mice: paw liftings can be observed beyond paw lickings. In this model nocifensive behavior was quantitatively evaluated by the duration of paw liftings and paw lickings, and “Composite Pain Score” (CPS=(1x duration of paw liftings+2x duration of paw lickings in seconds)/duration of examination period in seconds) was calculated [130]. PACAP-38 (2 μM, 100 μl) or its solvent (saline) was also injected intraplantarly 5 minutes before formalin administration. To study the involvement of specific receptors in the PACAP-induced effects, PAC1

receptor-selective M65 [1] or the VPAC1/VPAC2 receptor antagonist [D-p-Cl-Phe6,Leu17]-VIP [104] was administered (40 μM 50 μl) into the plantar region 5 minutes prior to PACAP-38 (4 μM 50 μl). PACAP-induced effects as well as the action of the antagonists were evaluated by comparison to the solvent (saline)-treated control group.

Measurement of mild heat injury-induced thermal hyperalgesia

The noxious heat threshold of Wistar rats was determined with a recently validated [7] increasing-temperature water bath. The equipment is suitable for the determination of the behavioral noxious heat threshold of rats defined as the lowest temperature at which the animal withdraws its hindpaw immersed into the water bath. After control threshold measurements rats were anaesthesized and one of the hindpaws was immersed in a constant, 51°C hot water bath for 20 seconds in order to evoke a mild burn injury. Following recovery from anaesthesia, heat threshold determinations were repeated 10 and 20 minutes after heat injury to confirm the development of hyperalgesia. PACAP-38 (2 μM 100 μl) or the same volume of saline was administered intraplantarly after the 20-minute measurement which was followed by repeated heat threshold measurements at 10-minute intervals. Thermal hyperalgesia was compared in percent to the initial control values at each time point. For studying the involvement of specific receptors, PAC1 receptor-selective M65 or the VPAC1/VPAC2 receptor antagonist [D-p-Cl-Phe$_6$,Leu$_{17}$]-VIP was administered (40 μM 50 μl) into the plantar region 5 minutes prior to intraplantar PACAP-38 (4 μM 50 μl).

Measurement of carrageenan-induced inflammatory mechanical allodynia

50 μl 3% carrageenan was injected into the plantar surface of one hindpaw of Wistar rats. Previous studies have revealed that this method is the most appropriate to study mechanosensitivity in inflammation models and that it reaches its maximum at 3 hours [8], so the measurements were performed before, 120 and 180 minutes after the induction of the acute inflammation. Mechanonociceptive threshold was determined by the Ugo Basile Dynamic Plantar Aesthesiometer. PACAP-38 (2 μM 100 μl) or the same volume of saline was administered intraplantarly 5 min before both measurements. The thresholds were compared to the initial control values and allodynia was expressed in percentage.

Investigation of the effect of PACAP-38 on basal mechano- and thermonociceptive thresholds

Before and 10 minutes after intraplantar injection of PACAP-38 (2 μM 100 μl) mechanonociceptive and thermonociceptive thresholds were measured in rats with dynamic plantar aesthesiometry and with increasing temperature water bath, respectively.

Investigation of acute visceral chemonocifensive behaviours: acetic acid-induced writhing test

Acetic acid (3% 200 μl) was injected intraperitoneally to CD1 mice to evoke abdominal constriction responses, which are typical nocifensive reactions [22,52,57]. The animals were placed in a transparent plastic box right after the challenge and their responses were counted during continuous observation for 20 minutes. PACAP-38 (100 μg/kg) or saline was administered subcutaneously 30 minutes before acetic acid treatment.

Measurement of mechanical hyperalgesia in traumatic mononeuropathy

The common sciatic nerve of anaesthesized mice was exposed unilaterally high in the thigh and 1/3–1/2 of the nerve trunk was carefully separated and tightly ligated. Then the wound was closed and the animals were allowed to survive for 7 days. Mechanonociception of the hindpaws was measured with dynamic plantar aesthesiometer and hyperalgesia was expressed in percent compared to the inital control values. PACAP-38 (10 or 100 μg/kg) or the solvent (saline) was administered intraperitoneally 30 minutes before measurement.

Electrophysiological measurement of primary afferents in the rat knee joint

These experiments were performed in cooperation with the Department of Physiology and Biophysics, University of Calgary. Acute synovitis was induced in the right knee joint by intra-articular injection of 2% kaolin followed by 2% carrageenan, total injected volume was 200 µl. The hip joint was immobilised by attaching a specially designed clamp to the femur at mid-thigh and connecting it to a stereotaxic frame. The hindpaw was placed into a plastic boot and the knee joint was rotated. Finally, a longitudinal skin incision was made along the medial aspect of the hindlimb and the resulting skin flaps were secured to a metal "O" ring to create a pouch which was filled with warm paraffin oil. The saphenous nerve was cut distal to the knee joint to prevent sensory input from the hindpaw. Axon bundles of the proximal nerve stump were hooked over a platinum electrode and electrical activity in the nerve was recorded. The conduction velocity of the recorded nerve fibres was ascertained by measuring the latency to an evoked potential produced by electrically stimulating the receptive field. Initially, 3 movement cycles were performed to acquire control baseline nerve activity. 100 µl PACAP was administered in two doses (0.2 and 2 µM) to the knee by close intra-arterial injection via the saphenous artery. Then the number of action potentials/movement was determined and the percent change in afferent activity following PACAP administration was calculated compared to control.

Statistics

Data for thermal and mechanical hyperalgesia/allodynia were analysed by one-way ANOVA followed by Dunnett's post test. For the electrophysiological data two-way ANOVA, for the formalin test and the writhing test Student's t-test was used. In all cases *$p<0.05$ and **$p<0.01$ was considered to be significant.

II.2. RESULTS

Inhibitory effect of intraplantar PACAP-38 on formalin-induced acute nocifensive behaviours

Nocifensive behavior expressed as composite pain score (CPS) and calculated from paw lickings and liftings was significantly inhibited by intraplantar injection of PACAP-38 both in the early phase (0-5 minutes) referring to acute chemonociception and in the late phase (20-45 minutes) evoked by inflammatory reactions. Local administration of the selective PAC1 receptor antagonist M65 5 minutes prior to PACAP-38 into the plantar region did not alter the PACAP-induced anti-nociceptive effect in either phase. Meanwhile, pre-injection of the VPAC1/2 receptor antagonist [D-p-Cl-Phe6,Leu17]-VIP abolished the PACAP-evoked inhibitory action on the CPS in phase II, but did not influence PACAP action in phase I.

Inhibitory effect of intraplantar PACAP-38 on mild heat injury-induced thermal hyperalgesia

Following the 51°C heat injury, the thermal thresholds dropped markedly, which maintained at an even level for at least 60 minutes. PACAP-38 administered after the 20-minute measurement markedly reduced heat injury-induced thermal hyperalgesia almost each time points as compared to the solvent-treated group. Intraplantar injection of the selective PAC1 receptor antagonist M65 5 minutes prior to the same dose of PACAP-38 into the plantar region did not alter the PACAP-induced anti-hyperalgesic effect, but the VPAC1/2 receptor antagonist [D-p-Cl-Phe6,Leu17]-VIP abolished the PACAP-evoked inhibitory action.

Inhibitory effect of intraplantar PACAP-38 on carrageenan-induced mechanical allodynia

We have found dropped mechanonociceptive thresholds in the control group 2 and 3 hours after carrageenan-injection. PACAP-38-treated group showed significant smaller changes in thresholds in both time points.

No effect of intraplantar PACAP-38 on mechano- and thermonociceptive thresholds

Intraplanar administration of PACAP-38 did not influence either the basal mechano-, or the thermonociceptive thresholds.

Peripheral inhibitory effect of PACAP-38 on acute visceral chemonociception

Subcutaneously injected PACAP-38 evoked significantly less acetic acid-induced writhing movements compared to the saline-treated control group. In this visceral chemonociception model PACAP also exerted an anti-nociceptive effect.

No effect of PACAP-38 on neuropathic mechanical hyperalgesia

7 days after nerve injury marked mechanical hyperalgesia developed in the hindpaw of mice, which was not inhibited significantly by any dose of the intraperitoneally injected PACAP-38. In this model PACAP-38 proved to be uneffective.

Sensitizing peripheral effect of PACAP-38 on afferent activity in inflamed knee joint

Kaolin-carrageenan-injection resulted in a significant increase of knee joint diameter. In the examined fibres local administration of PACAP-38 caused a transient, but significant increase in the afferent activity. This sensitizing effect was dose-dependent, the smaller dose did not evoke changes in mechanosensitivity on joint afferents.

II.3. SUMMARY AND CONCLUSIONS

Although immunolocalization of PACAP-38 has been described in capsaicin-sensitive neurons [69,70], there are only few scattered data concerning its role in nociception focusing only on its central effects. This study provides the first results on the peripheral actions of PACAP-38 on nociceptive processes. We have shown that peripherally administered PACAP-38 inhibits acute somatic and visceral chemonociception, as well as inflammatory mechanical allodynia and heat injury-induced thermal hyperalgesia in both rats and mice. On the contrary, it did not alter neuropathic mechanical hyperalgesia in the mouse paw and even induced mechanical sensitization of rat knee joint afferents.

PACAP acts on a family of three $G_{s/q}$ protein-coupled receptors: the PAC1 receptor to which PACAP has much higher affinity than VIP, and the VPAC1/VPAC2 receptors to which both PACAP and VIP bind similarly [54,104,127]. These receptors are connected to G_s/G_q proteins, and although they are coupled the several signal transduction pathways, all mechanisms increase intracellular cAMP and Ca^{2+} concentration leading to neuronal activation. This mechanism underlies the direct sensitising effect of PACAP-38 on afferent fibres found in the knee joint with electrophysiological experiments, but contradicts the peripheral anti-nociceptive and anti-hyperalgesic actions we observed in other models.

The strong upregulation of PACAP in dorsal root ganglia following adjuvant-induced inflammation suggests a role for this peptide in inflammatory pain conditions, particularly during chronic processes [138]. PACAP functions as an immuno-modulator and the majority of the studies report on its anti-inflammatory actions [13,51,58,62] via inhibiting the production of inflammatory cytokines (TNFα, IL-6, IL-12), chemokines, transcriptional factors and other mediators [13,51,58,62]. PACAP also inhibits T cell proliferation, several macrophage-functions and chemotaxis in a VPAC1 receptor-mediated manner [14,15]. We have recently shown that not only the cellular, but also the acute neurogenic components of the inflammatory reactions are decreased by systemic PACAP application. We have also provided direct *in vitro* evidence that PACAP-38 inhibits the release of pro-inflammatory sensory neuropeptides (SP and CGRP) from the peripheral terminals of capsaicin-sensitive sensory fibres which serves as an explanation for its ability to decrease plasma protein extravasation of exclusively neurogenic origin [35].

Although the precise mechanism for the observed anti-nociceptive and anti-hyperalgesic actions of PACAP-38 is not known, our results with selective PAC1 and VPAC1/VPAC2 receptor antagonists clearly showed that the peripheral inhibitory actions in the second phase of the formalin test as well as

in the heat injury-induced thermal hyperalgesia model are mediated by the activation of VPAC receptors. This is not likely to be a direct inhibitory action on the nociceptors, since the increased intracellular cAMP and Ca^{2+} levels lead to neural stimulation [54,105,117,118,127]. Meanwhile, elevation of intracellular cAMP attenuates the release of inflammatory mediators from mast cells and granulocytes [53,133]. The observed peripheral anti-nociceptive and anti-hyperalgesic actions of PACAP in the acute inflammation-associated models might be explained by the decreased release of the pro-nociceptive neuropeptides as well as other sensitizing agents (bradykinin, prostaglandins, leukotriens, serotonin, etc.) from cellular sources. The finding that neither the basal mechanical nor the thermal nociceptive thresholds were altered by local PACAP injection suggests that it does not inhibit voltage-gated sodium channels, therefore local anaesthetic-like effect is not involved in these peripheral anti-nociceptive actions.

Surprisingly, PACAP exerted a marked anti-nociceptive effect in phase I of the formalin test. This inhibition was not altered either by PAC1 or VPAC receptor antagonism, therefore, nonspecific, non receptor-mediated mechanisms are likely to be involved, but the existence of a presently unknown receptor for PACAP-38 or an overlapping action on other inhibitory receptors such as cannabinoid, opioid or somatostatin receptors cannot be excluded either [71].

In contrast to the observed inhibitory effects of PACAP-38 on visceral and somatic nociceptive reactions in the paw, in the acutely inflamed rat knee joint electrophysiological results clearly showed a sensitizing action. Thus, it is very likely that PACAP-38 also activates VPAC1/VPAC2 receptors located on nociceptive nerve terminals within the articular capsule which leads to enhanced joint mechanosensitivity. The intracellular mechanisms which could explain this sensitizing action might be related to cAMP accumulation in response to adenylate cyclase activation and enhanced protein kinase A activity, but phospholipase C activation can also be involved [105,117,118].

Besides these direct neural mechanisms of action, VPAC receptors have also been shown on human synoviocytes [120]. Fibroblast-like and macrophage-like synoviocytes are unique cells in the joints which are able to secrete several sensitizing inflammatory mediators including IL-1, PGE_2 and TNFα [141]. Furthermore, histamine released from synovial mast cells also acts as an algogenic substance in the joints. Evidence has been given for the ability of VIP to cause mast cell degranulation [102]. Therefore, an indirect action of PACAP to activate synoviocytes and mast cells and inducing the release of sensitizing substances in the acutely inflamed joints is also possible [65].

We found no change in mechanical hyperalgesia after systemic administration of PACAP in mononeuropathy model, despite electrophysiological studies have shown its ability to enhance activity of dorsal horn neurons in rat experimental mononeuropathy [18]. Although in this model PACAP-38 was injected intraperitoneally., this large peptide is very unlikely to penetrate through the blood-brain barrier, so at the peripheral level its significance could not been supported.

In conclusion, these results represent the first data for the peripheral actions of PACAP-38 on nociceptive transmission. These effects seem to be divergent depending on the mechanisms of nociceptor activation and the targets of PACAP actions. In acute visceral and somatic inflammatory pain models PACAP exerts anti-nociceptive, anti-hyperalgesic and anti-allodynic effects, while it causes mechanical sensitization in the acutely inflamed knee joint. Further studies are needed to completely elucidate both neural and non-neural factors in order to define the exact molecular mechanisms of PACAP effects on peripheral nociception.

III. STATIC MAGNETIC FIELD-INDUCED ANTI-NOCICEPTIVE EFFECT AND THE INVOLVEMENT OF CAPSAICIN-SENSITIVE SENSORY NERVES IN THIS MECHANISM

The effects of static magnetic field (SMF) on several behavioural patterns and neural functions such as induction of locomotor activity [44], conditioned taste aversion [74], and vestibular activation [103] have been described recently. Data available on the actions of SMF on nociceptive processes are contradictory [12,16,43,49,86,88,101]. The reason for this contradiction might be differences between the species and the characteristics of the magnetic field (e.g. intensity) and the duration of the exposure.

The SMF generating device used in the experiments was developed, optimized and validated by Dr. János László [56]. This device contains two matrices with permanent neodymium-iron-boron (NdFeB) cylindrical magnets (5 mm radius, 10 mm height) with alternating poles. These are the strongest rare-earth metal magnets. The individual magnets has grade N50, their remanent magnetic induction is 1.47 T. Two adjacent individual magnets have altered polarity as well as in the opposite position in the matrix with the same axis and orientation. These parameters result in a characteristic pattern of the magnetic field, which is described as an optimized static magnetic field, oSMF [56].

Although the mechanisms by which SMF mediates its great variety of actions are unknown [59,94], modification of the endogenous opioidergic system and ion channel conduction properties have been suggested [48,92,93]. Several data indicate the ability of relatively weak SMF to diminish neural excitability by the inhibition of Ca^{2+} and Na^{+} currents [92,93,134].

As previously described, capsaicin-sensitive, TRPV1 receptor-expressing sensory nerves play an important role in several inflammatory and nociceptive processes via the release of pro-inflammatory/pro-nociceptive sensory neuropeptides such as SP and CGRP into the innervated area [60,109]. Meanwhile, somatostatin is also released from the capsaicin-sensitive subpopulation of primary afferents, reaches the circulation and it is able to elicit systemic anti-inflammatory and anti-nociceptive actions [38,82,111,112].

The TRPV1 receptor is a non-selective cation channel, which is activated by exogenous vanilloid compounds (e.g. capsaicin or resiniferatoxin: RTX), as well as noxious heat and several endogenous chemical stimuli, such as protons, bradykinin, and leukotriens produced in the inflamed tissues [107,109]. The function of capsaicin-sensitive primary sensory fibres can be selectively blocked by pretreatment with repeated high doses of TRPV1 agonists (capsaicin or RTX), this mechanism is called desensitization [6,39,113]. After repeated high doses of RTX treatment intracellular Ca^{2+} concentration increases and Ca^{2+} accumulates in the mitochondria evoking metabolic damage of the cells. This is accompanied by ultrastructural changes like disorganized, swollen mitochondria [108].

The aim of the present series of experiments was to elucidate the effects of oSMF exposure on acute visceral and somatic chemonociception, as well as on acute inflammatory nocifensive behaviours and inflammatory mechanical hyperalgesia in mice using an optimized SMF generating device. Furthermore, the involvement of capsaicin-sensitive sensory fibres in the oSMF-induced actions was also investigated.

III.1. EXPERIMENTAL MODELS AND METHODS

Animals

Experiments were performed on Balb/c and CFLP mice bred and kept under pathogen free conditions at 24-25°C and provided with standard chow and water *ad libitum* in the Laboratory Animal Center of the University of Pécs and in the Semmelweis University, respectively.

The optimized static magnetic field

One matrix was positioned above and one below a special plastic cage, where the mice were placed. Mice in the control group were put into a similar cage without magnetic field exposure (sham box). The size of the cage was 140*140*46 mm (length*width*height).

Writhing test

Visceral nociception was elicited in CFLP mice by intraperitoneal injection of 200 μl 0.6% acetic acid or 2% $MgSO_4$ [135]. As a result of chemical irritation of the peritoneum abdominal constrictions (writhing movements) were observed as typical nocifensive behaviours. Two or three mice were placed in the cage at the same time, the cage was put into the oSMF 5 minutes before the injection and the animals were left there throughout the whole experimental period. The number of writhings was counted during the 0-5, 5-20 and 20-30 minute time intervals. Mice in the control group were placed in the same box without oSMF.

Resiniferatoxin-induced mechanical hyperalgesia

Measurements were performed before and 30 minutes after subcutaneous injection of 20 μl ultrapotent TRPV1 receptor agonist RTX (0.1 μg/ml) into the plantar surface of the left hindpaw of Balb/c mice. Hyperalgesia in each mouse was expressed as percentage decrease of the mechanonociceptive thresholds compared to the control values. Mice were placed into the oSMF for 30 minutes directly before measurements. In the control group animals were placed into the same box without SMF.

Formalin-induced acute nocifensive behaviour

50 μl 2.5% formalin injected subcutaneously into the plantar surface of one hindpaw of Balb/c mice induced nocifensive reactions in two phases, the first of which (0-5 minutes) is thought to be due to a direct chemonociceptive effect of formalin, while the second one (20-45 minutes) is mainly mediated by inflammatory reactions [123]. Nocifensive behaviour was quantitatively evaluated by the total duration of paw lickings in each examination period [8]. Mice were placed into the oSMF for 5 minutes before and throughout the whole experimental period. Animals in the control group were put into the same box without SMF.

Carrageenin-induced inflammatory mechanical hyperalgesia

50 μl 3% carrageenan was injected into the plantar surface of one hindpaw of Balb/c mice. Mechanonociceptive threshold of the hindpaws was determined by Ugo Basile Dynamic Plantar Aesthesiometer as earlier described. Measurements were performed before and 3 hours after subcutaneous injection. Hyperalgesia was expressed in percent of the mechanonociceptive thresholds compared to the control values. Mice were placed into the SMF for 30 minutes before measurements. In the control group animals were placed into the same box without SMF.

Desensitization of capsaicin-sensitive afferents with resiniferatoxin pretreatment

The function of capsaicin-sensitive primary sensory fibres was selectively abolished by repeated pretreatment with high doses of TRPV1 agonist RTX (30, 70 and 100 μg/kg s.c. on three consecutive days 5 days before the study). RTX-pretreated mice were examined in the formalin test and the in the carrageenan-induced acute inflammation model.

Rotarod test

Mice were studied in Ugo Basile RotaRod device in order to examine if oSMF influences coordination or motor function [47]. Their RotaRod performance was measured by the duration of time (seconds) spent on the rotating drum. They were examined before and after they had been placed into oSMF or in the control group into the same box without SMF for 30 minutes. Each measurement was repeated 3 times and their means with standard errors (s.e.m.) were calculated.

Statistics

For statistical evaluation of all data one-way ANOVA followed by Bonferroni's modified t-test was used. In all cases comparing two different group *$p<0.05$ was considered to be statistically significant.

III.2. RESULTS

Inhibitory effect of oSMF on acute visceral chemonocicepction

Intraperitoneally injected $MgSO_4$ induced less nocifensive behaviours than acetic acid. Exposure of mice to oSMF resulted in significantly fewer abdominal contractions evoked by 0.6% acetic acid compared to the control group. In the case of $MgSO_4$ stimulation the oSMF-induced anti-nociceptive effect in the second and third examination periods also proved to be statistically significant, but the degree of the inhibition was smaller than in the case of acetic acid.

Inhibitory effect of oSMF on resiniferatoxin-induced mechanical hyperalgesia

The TRPV1 receptor agonist RTX decreased mechanical touch sensitivity of the hindpaw in the control group 30 minutes after its intraplantar injection. In mice which were placed into oSMF for the whole 30-minute period between RTX administration and measurement, significantly smaller hyperalgesia developed than in animals kept in the control box.

Inhibitory effect of oSMF on formalin-induced acute somatic nociception

The number of intraplantar formalin-induced paw lickings were significantly smaller in mice placed into the oSMF 5 minutes before and during the whole examination period both in the early phase (0-5 minutes) of the test referring to direct chemonociception and in the late phase (20-45 minutes) evoked by acute inflammatory reactions.

Inhibitory effect of oSMF on carrageenan-induced inflammatory mechanical hyperalgesia

The mechanonociceptive threshold was measured 3 hours after carrageenan injection and compared to the initial, preinjection values. In mice which were placed into oSMF for a 30-minute period before measurement significantly smaller hyperalgesia developed compared to control animals not being in the magnetic field.

Effect RTX-pretreatment on oSMF-induced anti-nociceptive action in the formalin test

In mice which were pretreated with RTX 5 days before the formalin test in order to inactivate capsaicin-sensitive sensory nerves, we found almost the same values as mesured in the study described above. However in that experiments oSMF had significant anti-nociceptive effects, in this case it did not induce significant inhibitory action in either phases.

Effect RTX-pretreatment on oSMF-induced anti-hyperalgesic action in the carrageenan model

Although in mice which were pretreated with RTX carrageenan-induced hyperalgesia was smaller, it did not prove to be statistically significant. The inhibitory action of oSMF observed in untreated mice was absent in the RTX-pretreated group.

No effect of oSMF on motoric coordination and function in mice

Placing the mice into oSMF for 30 minutes did not alter their RotaRod performance compared to the control group before and after being in the same box for the same time without SMF. No significant differences could be detected either between the two groups or between respective performances before and after the 30-minute period.

III.3. SUMMARY AND CONCLUSIONS

These results demonstrate that acute exposure of mice to oSMF exerts inhibitory effects on visceral/somatic chemonociception and inflammatory nociception/hyperalgesia. The oSMF-induced inhibition was more pronounced on the abdominal writhing response evoked by acetic acid than by $MgSO_4$. This difference might be explained by distinct pain-producing mechanisms of these chemicals: the nocifensive response elicited by $MgSO_4$ may be due to direct stimulation of visceral chemonociceptors, while an acute inflammatory reaction is likely to be involved in the acetic acid-induced behaviour [30].

Phase I of the formalin test is due to the direct chemical stimulatory action of the compound, in phase II inflammatory mechanisms are involved [123]. Nocifensive reactions in phase II, but not in phase I were reduced in control (sham box-exposed) mice after selective inactivation of capsaicin-sensitive sensory fibres by RTX pretreatment. Therefore, SP and CGRP released from these nerves are likely to be involved in the development of this inflammatory reaction. The oSMF exposure diminished nocifensive behaviours in both phase I and phase II. The extent of the inhibition in the late phase was similar to that observed after RTX pretreatment. This inhibition was prevented after the destruction of peptidergic sensory fibres by RTX. One potential mechanism for the inhibition in phase I might be a direct inhibition of sodium influx and action potential generation at the level of capsaicin-sensitive nerve terminals. In the SMF-induced inhibitory effect in phase II decreased Ca^{2+} influx and consequently diminished sensory neuropeptide release mediating the local neurogenic inflammatory response can also be considered. This theory is supported by data on the ability of oSMF to diminish neural excitability by the inhibition of Ca^{2+} and Na^{+} currents [92,93,134].

Carrageenan produces a subacute local inflammation in which oedema and mechanical hyperalgesia reaches its maximum 3 hours after injection [8]. Both neurogenic and non-neurogenic mechanisms with several mediators such as inflammatory neuropeptides, cytokines and cyclooxygenase products are involved in this process [20,84]. Carrageenan-induced mechanical hyperalgesia was smaller, but not significantly decreased after RTX pretreatment, which suggests that capsaicin-sensitive nerves do not play a predominant role in this type of oedema formation. Similarly to the other models, SMF-exposure exerted a marked inhibitory action on this inflammatory mechanical hyperalgesia, which was also prevented after the destruction of peptidergic afferents by RTX pretreatment.

SMF also diminished inflammatory mechanical hyperalgesia induced by intraplantar injection of the TRPV1 receptor agonist RTX. In response to the activation of this cation channel on capsaicin-sensitive sensory nerve terminals by RTX, pro-inflammatory neuropeptides such as SP and CGRP are released in the innervated area, which produce a local neurogenic inflammatory reaction in the plantar surface of the hindpaw with a consequent drop of the mechanonociceptive threshold. Since the development of mechanical hyperalgesia in this model involves peripheral mechanisms, one explanation for the anti-nociceptive action of oSMF might be the inhibition of activation-induced cation influx into the nerve terminals leading to a decreased release of pro-inflammatory/pro-nociceptive neuropeptides. This theory is supported by data on the ability of SMF to diminish neural excitability by the inhibition of Ca^{2+} and Na^{+} currents [92,93,134]. Besides this, neither inhibition of central sensitization nor decreased synaptic transmission can be excluded.

Exposure of mice to oSMF for 30 minutes did not alter their RotaRod performance, therefore impairment of motor function cannot be considered as a factor influencing their nocifensive behaviours.

In conclusion, this study is the first to provide experimental evidence for the involvement of capsaicin-sensitive sensory nerves in the oSMF-induced anti-nociceptive action in different animal models. The mechanism is not completely known yet and precise elucidation of these processes needs further investigation.

SUMMARY OF THE NOVEL FINDINGS

1. With the help of the selective somatostatin sst_4 receptor agonist J-2156 we have provided evidence for the anti-inflammatory and anti-allodynic/anti-hyperalgesic role of this receptor in a variety of mouse and rat models. It is particularly worth emphasizing that in both species this compound was able to effectively inhibit the neurogenic components of the inflammatory processes which are involved in the pathological mechanisms of several diseases and for the treatment of which the presently available drugs are not satisfactory. Furthermore, it is also important that this sst_4 receptor agonist diminished mechanical allodynia in the traumatic neuropathy model, for which the classical non-steroidal analgesics or opioid compounds are ineffective. Although certain anti-epileptics and anti-depressants provided significant pain-relief in certain neuropathic pain conditions, the treatment of these problems is still not completely resolved. Our present findings are the first which identify and validate a novel target molecule, the sst_4 receptor, expressed both on the sensory nerve terminals and inflammatory cells. Based on these data, stable, selective, primarily non-peptide agonists might open promising perspectives for the development of an effective, broad-spectrum anti-inflammatory and analgesic drug group with a completely new mechanism of action.

2. Our present findings are the first for the peripheral actions of PACAP-38 on nociceptive processes. Electrophysiological experiments showed sensitizing effect of locally administered PACAP-38 in the rat knee joint similarly to the previously described actions of VIP. On the contrary, in several, mainly inflammation-related rodent models we have found peripheral anti-nociceptive and anti-hyperalgesic effects of PACAP which are mediated through the VPAC receptors. This contradiction could be explained by the fact that increased intracellular cAMP level induced by VPAC receptor activation results in neuronal excitation, but diminishes the production and release of inflammatory mediators and cytokines. Therefore, the overall inhibitory action on nociception is not likely to be due to a direct effect on the sensory nerve terminals, but much more to an indirect mechanism via its anti-inflammatory actions. Evaluation of the practical significance of these findings as well as potential drug developmental perspectives of PACAP needs further investigations.

3. Besides the pharmacological approaches, in the third part of the work an alternative possibility for analgesic therapy was examined with the help of an optimized static magnetic field (oSMF). Exposure of mice to oSMF proved to be anti-nociceptive and anti-hyperalgesic in various mouse models of nociception and inflammatory hyperalgesia. The involvement of capsaicin-sensitive sensory nerves was evidenced in the observed inhibitory action of oSMF. Although further studies are required to elucidate the precise molecular mechanism, inhibition of voltage-gated cation channels (Na^+, Ca^{2+}) or the function of the TRPV1 receptor might be considered as potential effects. Through these actions, oSMF might inhibit action potential generation and propagation as well as the release of pro-nociceptive sensory neuropeptides. Understanding the static magnetic field-induced neuronal mechanisms could promote its therapeutical use and show interesting directions for non-pharmacological analgesic interventions.

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