

PHD THESIS

**ROLE OF NEUROGENIC COMPONENTS IN INFLAMMATORY
DISEASES OF THE SKIN, JOINT AND COLON**

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Introduction

Capsaicin-sensitive nociceptive afferent neurons. Nociceptive afferent neurons transport information into the central nervous system about potentially noxious stimuli.

It has been known for a long time that antidromic electrical stimulation of the peripheral stumps of afferent nerves of the skin, exteroceptive mucosal areas and visceral organs (Lundberg et al., 1984; Szolcsányi, 1984) leads to vasodilatation (Bayliss, 1923; Langley, 1923) and subsequent plasma extravasation. This phenomenon is called neurogenic inflammation (Jancsó et al., 1967; Jancsó et al., 1968). The major subpopulation of nociceptive nerve fibres associated with this response is selectively excited and, in high dose, desensitized by capsaicin, and is now called “capsaicin-sensitive afferents” (Szolcsányi, 1982; Szolcsányi, 1996). Neurogenic inflammation is mediated by sensory neuropeptides released from capsaicin-sensitive nerve fibres. Capsaicin-sensitive afferents also release neuropeptides associated with anti-inflammatory actions (somatostatin, galanin, PACAP-38).

Neurogenic inflammation may contribute to the pathomechanism of several diseases like bronchial asthma (Bertrand et al., 1993; Germonpré et al., 1997), allergic rhinitis (Bertrand et al., 1993; Quartara & Maggi, 1998), conjunctivitis and dermatitis (Gutwald et al., 1991), ekzema (Naukkarinen et al., 1996), rheumatoid arthritis (Levine et al., 1985), migraine (Buzzi & Moskowitz, 1990; Williamson & Hargreaves, 2001) and inflammatory bowel disease (Renzi et al., 2000). Neurogenic component of these conditions can not be relieved by orthodox non-steroidal anti-inflammatory agents (Jancsó-Gábor & Szolcsányi, 1972; Helyes et al., 2001).

TRPV1 capsaicin receptor. Recently, the cloning of capsaicin receptor succeeded (Caterina et al., 1997; Caterina & Julius, 2001; Clapham et al., 2003). Homology was found with transient receptor potential (TRP) ion channels and the receptor was named transient receptor potential vanilloid 1 (TRPV1) (Gunthorpe et al., 2002).

TRPV1 receptor is a non-selective cation channel and can be activated by noxious heat (over 43 °C), protons (Tominaga et al., 1998), exogenous compounds (capsaicin, resiniferatoxin, piperin, zingerone, gingerol, ethanol) (Szolcsányi, 1983; Szállási & Blumberg, 1989; Patacchini et al., 1990; Szállási & Blumberg, 1990; Caterina et al., 1997; Liu et al., 2000; Dedov et al., 2002), endogenous compounds (anandamide, lipoxygenase products, N-oleoyl-dopamine) (Hwang et al., 2000; Trevisani et al., 2002; Chu et al., 2003).

Pro-inflammatory sensory neuropeptides. Main members of pro-inflammatory sensory neuropeptides include tachykinins (substance P, neurokinin A, neurokinin B) and CGRP.

Main sources of tachykinins are capsaicin-sensitive sensory neurons, but they can also be found in some non-neurogenic elements. Tachykinins activate G protein coupled receptors (NK1, 2, 3). SP, NKA and NKB are all full agonists of NK1, 2 and 3 receptors. SP has the highest affinity to NK1, NKA to NK2 and NKB to NK3 receptors.

Tachykinins released in the dorsal root of the spinal horn contribute to pain sensation. They also modulate cholinergic and adrenergic neurotransmission (Grant, 2002). Tachykinins induce endothel-dependent vasodilation. They facilitate proliferation, migration of endothelial cells and angiogenesis (Maggi, 1995). Tachykinins evoke plasma protein extravasation. Smooth muscle contraction can be produced by tachykinins via release of other mediators and mast cell degranulation. They can also contract smooth muscles directly. SP stimulates the secretion of salivary, intestinal glands and the pancreas (Lembeck & Starke, 1968; Konturek et al., 1981).

CGRP belongs to the calcitonin/CGRP peptide family. CGRP acts via G protein coupled receptors. CGRP has mostly extraneural effects. It facilitates the release of acetylcholine from the myenteric plexus of the guinea pig ileum, but inhibits the release of noradrenaline and ATP. CGRP applied intravenously causes hypotension, tachycardia, enhanced coronary and mesenteric blood flow due to vasodilation. This vasodilation is independent of endothelial cells. CGRP induces smooth muscle relaxation in several organs (Maggi, 1995). CGRP exerts positive chronotropic and inotropic actions on the heart (Tippins et al., 1984). It facilitated attachment of neutrophils to vascular endothelium (Hartung & Toyka, 1989). However, CGRP diminished maturation of pre-B lymphocytes, their immunoglobuline production and proliferation of T lymphocytes. CGRP inhibited the secretion of pro-inflammatory cytokines (IL-1, IL-12) by macrophages, but facilitated the production of the anti-inflammatory IL-10. These data demonstrate that CGRP may have anti-inflammatory properties when disease is mainly mediated by lymphocytes and macrophages.

PACAP-38 and capsaicin-sensitive primary afferent neurons. Pituitary adenylate cyclase activating polypeptide (PACAP) is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family.

Two types of PACAP binding sites were evidenced in different tissues. Type I binding sites represent PAC1 receptors which are specific for PACAP (Pisegna & Wank, 1993). Type II binding sites represent VPAC1 and 2 receptors which bind PACAP and VIP, too (Ishihara et al., 1992; Vaudry et al., 2000). The receptors of PACAP are G protein-coupled receptors. PAC1 receptor is the predominant form in the brain. VPAC1 receptor mRNAs were identified in the cerebral cortex and the hippocampus. VPAC2 mRNAs were found in the thalamus, the suprachiasmatic nucleus, the central nucleus of the amygdala and the pontine nucleus (Usdin et al., 1994; Sheward et al., 1995). Messenger RNAs of PACAP binding receptors were detected in most endocrine glands

(Usdin et al., 1994; René et al., 1996). They were also demonstrated in the alimentary canal and accessory glands, the respiratory tract, the cardiovascular system, the kidneys, white fat and skeletal muscles (Ishihara et al., 1992; Usdin et al., 1994; Wei & Mojsov, 1996; Wong et al., 1998). Macrophages and lymphocytes express PACAP receptors, too (Delgado et al., 1996; Johnson et al., 1996).

PACAP occurs in the central and peripheral nervous system, endocrine glands, cardiovascular, respiratory and gastrointestinal tracts. PACAP has diverse actions in the central and peripheral nervous systems and also in peripheral tissues (Somogyvári-Vigh & Reglódi, 2004; Zhou et al., 2002).

In sensory ganglia, nerve plexus of inner organs (Skakkebaek et al., 1999; Hannibal & Fahrenkrug, 2000), pineal gland (Moller et al., 1999), skin and in teeth (Ichikawa & Sugimoto, 2003) PACAP-38 coexists with CGRP. The coexistence of PACAP and SP has also been reported (Strange-Vognsen et al., 1997; Mirabella et al., 2001).

Aims

1. We would like to clarify whether PACAP-38 can be released from capsaicin-sensitive primary afferents in response to electrical and chemical stimuli under *in vitro* conditions.
2. We also would like to examine if PACAP-38 can be released from capsaicin-sensitive afferents *in vivo* and if it can reach the systemic circulation.
3. Our aim was to study the effect of systematically applied PACAP-38 on different animal models of inflammatory disease. These include both neurogenic and mixed type inflammatory conditions.
4. We would like to analyze the participation of capsaicin-sensitive nerve terminals and TRPV1 receptors in the sensitizing effect of PMA-induced ear inflammation.
5. We would like to investigate the role of prostanoids and IL-1 β in the sensitizing effect of PMA-evoked ear inflammation.
6. Our aim was to determine the function of capsaicin-sensitive afferents and TRPV1 receptors in DSS-induced colonic inflammation.

Effect of PACAP-38 on sensory neuropeptide release, acute neurogenic and non-neurogenic inflammatory processes in rats and mice

Numerous studies suggest that PACAP has anti-inflammatory actions (Abad et al., 2001; Gomariz et al., 2006). Relatively little is known about the actions of PACAP on neurogenic components of

inflammation. There are two sources of PACAP in the immune system: neurons innervating the lymphoid organs and immune cells themselves. Most important anti-inflammatory effect of PACAP is direct inhibition of macrophage activity (Delgado et al., 1999).

Methods

Measurement of capsaicin-evoked and electrically-induced sensory neuropeptide release from isolated rat tracheae. Tracheae of anaesthetized rats were removed, placed into organ baths (1.8 ml) and perfused (1 ml/min) with pH 7.2 oxygenized Krebs solution for 60 min at 37 °C. Pre-stimulated, stimulated and post-stimulated fractions were collected, 8 min each. Electrical field stimulation (EFS; 40 V, 0.1 ms, 10 Hz for 120 s; 1200 pulses) was performed or capsaicin (10^{-6} M) was added into the medium. To examine the effect of PACAP-38 on the release of the sensory neuropeptides it was added into the incubation medium.

PACAP-38, CGRP, substance P and somatostatin concentrations were determined from the incubation medium by radioimmunoassay (RIA). The tracers were mono- 125 I-labelled peptides. Synthetic peptides were used as standards. The assay was prepared in 1 ml 0.05 mol/l, pH 7.4 phosphate buffer containing 0.1 mol/l sodium chloride, 0.25% BSA and 0.05% sodium aside. The antiserum (100 μ l, 1:10000), the RIA tracer (100 μ l, 5000 cpm/tube) and the standard or unknown samples (100 μ l) were put into the assay buffer. After 48-72 h incubation at 4 °C, the antibody-bound peptide was separated and radioactivity was measured.

Identification of PACAP-38 from the plasma with mass spectrometry. Identification of PACAP-38 from plasma samples in comparison to standard PACAP-38 peptide was performed with matrix-assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometry. One μ l of PACAP-38 standard and the naïve rat serum was mixed with the same volume of saturated matrix solution. The ions were accelerated under delayed extraction conditions (200 ns) in positive ion mode with a voltage of 20 kV.

Systemic stimulation of capsaicin-sensitive afferents *in vivo*. Resiniferatoxin was administered (3 μ g/kg, i.v.) into tail veins of anaesthetized rats. Blood samples were taken by cardiac puncture 5 min after RTX injection.

Antidromic stimulation of the sciatic nerve. Peripheral stumps of cut sciatic nerves of anaesthetized rats were stimulated (20 V, 0.5 ms, 5 Hz, 5 min) (Szolcsányi et al., 1998b). Guanethidine (8 mg/kg, i.p.) was administered 1 h before nerve excitation. Pipecuronium bromide (200 μ g/kg, i.v.) was injected and positive pressure ventilation was carried out. Blood samples were taken by cardiac puncture 5 min after stimulation.

Plasma preparation for radioimmunoassay. Blood samples (6 ml per animal) were mixed with EDTA (12 mg) and aprotinin (1200 U). Following centrifugation (2000 rpm for 10 min at 4 °C) the peptide content was extracted by 3 volumes of absolute alcohol. After second centrifugation samples were dried under nitrogen flow and resuspended in 300 µl assay buffer (Jakab et al., 2004).

Measurement of mustard oil-induced neurogenic oedema formation in the mouse ear. Ear swelling was evoked by topical application of 10 µl of 1% mustard oil dissolved in liquid paraffin on the inner and outer surfaces of both ears at the beginning of the experiment and 1 h later. Ear thickness was measured with an engineers' micrometer (0.01 mm accuracy). PACAP-38 (10, 100 and 1000 µg/kg in 200 µl saline, i.p.) was administered 15 min before both mustard oil smearing.

Measurement of mustard oil-induced acute neurogenic inflammation in the skin of the rat hindpaw. Both hindlegs of anaesthetized male Wistar rats were acutely denervated. Acute neurogenic inflammation was evoked by 1% mustard oil dissolved in paraffin oil. Extravasation of plasma albumin was measured by the Evans blue leakage method. Rats were killed by exsanguination 20 min after mustard oil application. PACAP-38 (100 µg/kg, i.p.) was administered 20 min before the induction of inflammation.

Measurement of acute neurogenic inflammation evoked by TRPV1 receptor agonists in the plantar skin of the rat hindpaw. Both hindlegs of anaesthetized rats were acutely denervated. Acute neurogenic inflammation was evoked by intraplantar injection of RTX (100 µl, 0.1 µg/ml) or capsaicin (100 µl, 100 µg/ml) into the left hindpaw. Extravasation of plasma albumin was measured by the Evans blue leakage method. Rats were exsanguinated 20 min after the application of RTX or capsaicin. PACAP-38 (10 µg/kg) or isotonic saline were administered i.p. 10 min before the induction of inflammation.

Measurement of carrageenan-induced oedema formation in the rat hindpaw. Carrageenan (50 µl, 3%) was injected intraplantarly into the left hindpaw of anaesthetized rats. The volume of the paw was measured with plethysmometry before and 60, 120, 180 min after carrageenan administration (Helyes et al., 2006). In a group of rats 10 µg/kg PACAP-38 was administered i.p. 10 min before the induction of the inflammation (Helyes et al., 2001; Helyes et al., 2006).

Results

PACAP-38 is released from sensory nerve endings of the isolated rat trachea. Capsaicin (10^{-6} M) and electrical field stimulation (EFS) evoked 27% and more than two-fold elevation of PACAP-38 release, respectively.

PACAP-38, but not PACAP-27 can be detected in the plasma with mass spectrometry. PACAP-38 could be identified in the plasma samples of naïve rats with mass spectrometry at the molecular weight signal of 4558.7 Da, representing the quasimolecular ion of PACAP-38 Na⁺ adduct (MW: 4558.7). PACAP-27 (MW: 3147.6) or its [M+Na]⁺ could not be detected.

PACAP-like immunoreactivity of the rat plasma increases in response to systemic stimulation of capsaicin-sensitive nerves. Stimulation of capsaicin-sensitive nerves by injection of RTX (3 µg/kg, i.v.) induced a 2-fold elevation of plasma PACAP-like immunoreactivity (PACAP-LI). Antidromic electrical excitation of the sciatic nerve slightly elevated PACAP-LI.

PACAP-38 inhibits capsaicin- and EFS-evoked substance P, CGRP and somatostatin release from isolated rat tracheae. Capsaicin (10⁻⁶ M) induced a 2.5-, 11- and 3-fold elevation of substance P, CGRP and somatostatin release, respectively. EFS (1200 pulses) evoked a 3-, 3.5- and 2.5-fold increase in the outflow of these peptides. PACAP-38 (20-2000 nM) significantly and concentration-dependently inhibited the release of all the three sensory neuropeptides in both cases.

Effect of PACAP-38 on acute neurogenic oedema of the mouse ear. PACAP-38 (10, 100 and 1000 µg/kg) significantly diminished mustard oil-induced ear swelling in a dose-dependent manner.

Effect of PACAP-38 on acute neurogenic inflammation in the skin of the rat hindpaw. PACAP-38 (100 µg/kg, i.p.) exerted significant, 37.8% inhibition on mustard oil-induced Evans blue dye accumulation in the skin of the acutely denervated rat hindpaw.

PACAP-38 inhibits capsaicin- and resiniferatoxin-induced acute neurogenic inflammation in the plantar skin of the rat hindpaw. PACAP-38 (10 µg/kg, i.p.) 10 min before the induction of the inflammation with 100 µl intraplantar RTX (0.1 µg/ml) or capsaicin (100 µg/ml) exerted 45.7% and 46.4% inhibition.

Effect of PACAP-38 on carrageenan-evoked acute paw oedema in the rat. Carrageenan-induced oedema formation was significantly inhibited by 10 µg/kg PACAP-38, i.p.

Discussion

We provided clear evidence that PACAP-38 is released from the stimulated peripheral terminals of capsaicin-sensitive afferents. Furthermore, we described a specific and sensitive radioimmunoassay technique for reliable determination of PACAP-LI in the rat plasma. With the help of this method we showed that PACAP-38 is released from capsaicin-sensitive afferents into the systemic circulation via TRPV1 receptor. We proved that PACAP-38 is able to inhibit the outflow of neuropeptides from sensory fibres. Based on this mechanism of action it is also able to effectively diminish neurogenic inflammatory response *in vivo* after systemic administration. We also show the

ability of systemically administered PACAP-38 to inhibit mixed-type oedema formation in the rat paw.

Systemic sensitizing effect of non-neurogenic inflammation on capsaicin-sensitive afferents in the mouse ear

Phorbol 12-myristate 13-acetate (PMA)-induced mouse ear inflammation has been commonly used as a model to study the effect of anti-inflammatory agents (Garrido et al., 2004; Huang et al., 2006). Neurogenic components were also evidenced in PMA-induced inflammation in murine ears by vanilloid-pre-treated animals.

The aim of the present work was to investigate the role of capsaicin-sensitive sensory nerve endings and TRPV1 receptors in the nerve-sensitizing effect of PMA-induced ear inflammation.

Methods

PMA and acetone treatment. Right ears of anaesthetized animals were smeared with PMA (2.5 µg) dissolved in acetone. Left ears were smeared with acetone. Separate groups of animals received acetone on both ears or PMA on the right ear and the left ear remained untreated.

Systemic resiniferatoxin pre-treatment. RTX stock solution (1 mg/ml) was dissolved in 96% ethanol, further dilutions were produced by saline. Anaesthetized TRPV1^{+/+} animals received 10, 30 and 100 µg/kg RTX s.c. into the neck region on three consecutive days. Experiments were carried out 14 days after RTX pre-treatment. The effect of pre-treatment was checked by the eye wiping test.

Local capsaicin treatment. Right or left ears of TRPV1^{+/+} animals were treated with 0.5% capsaicin dissolved in 70% ethanol. Contralateral ears were smeared with ethanol. Capsaicin treatment was performed 5 times in every second hour and this procedure was repeated on the consecutive day. Animals were recruited to the experiment after 4 days (Gábor & Rázga, 1992).

Ibuprofen treatment. In a separate group of wild-type mice, 70 mg/kg ibuprofen sodium dissolved in saline was injected i.p. 45 min before the PMA-acetone treatment and this procedure was repeated in the 6th and 12th hours.

Anti-IL-1β antibody treatment. Five µg of polyclonal goat anti-mouse IL-1β IgG was administered i.v. to a group of mice. An hour after the injection animals received PMA-acetone treatment and ear thickness was checked as discussed below.

Measurement of ear oedema. Ear thickness was measured before smearing with either acetone or PMA and after the treatments in different time points as described previously.

Myeloperoxidase assay. Samples were collected 12 hours after acetone and PMA challenge and stored at -20 °C. Ears were weighed, chopped into small pieces and homogenised. The homogenate was centrifuged at 10000 g, 4 °C for 10 minutes and 500 µl of the supernatant was collected. Myeloperoxidase activity was determined in 96 well microtitre plates using 3, 3', 5, 5'-tetramethylbenzidine at room temperature.

Histology. Extent of oedema, formation of microabscesses after necrosis of hair follicles and sebaceous glands and number of accumulated leukocytes were scored.

Measurement of IL-1 β concentration. Samples were weighed, put into RPMI medium containing phenylmethylsulfonyl fluoride (1 ml each) and chopped into small pieces. Ears were homogenized and centrifuged at 10000 g, 4 °C for 10 minutes and 500 µl of supernatants were collected. IL-1 β content was determined by BD OptEIA ELISA set.

Results

Effect of genetic lack of TRPV1 receptor, systemic RTX pre-treatment, local capsaicin desensitization and ibuprofen treatment on PMA- and acetone-induced ear oedema. If the right ears were treated with PMA, acetone treated left ears produced approximately 85% increase of thickness within 12 hours both in TRPV1^{+/+} and ^{-/-} animals. In the first 4 hours these acetone treated ears did not show detectable swelling. PMA induced 163-196% ear swelling both in TRPV1^{+/+} and TRPV1^{-/-} mice.

Systemic pre-treatment of TRPV1^{+/+} animals with resiniferatoxin ameliorated contralateral acetone-induced ear swelling. PMA-induced ear oedema formation was inhibited by 30-50% particularly in the early phase (0-6 h).

Only 12-20% increase in ear oedema was observed if both ears were treated with acetone. If the left ear was not treated with acetone, PMA treatment on the right side induced about 30% increase in ear thickness on the untreated left side both in TRPV1 receptor knockout and wild type mice.

Neither genetic lack of TRPV1 receptors nor systemic RTX pre-treatment produced significant changes in bilateral acetone treatment-induced ear oedema.

In a separate animal group local capsaicin-desensitization was performed on the acetone treated left side of TRPV1^{+/+} mice. Acetone evoked only moderate ear swelling. If local capsaicin desensitization was fulfilled on the right, PMA-treated ear, PMA-evoked early ear oedema was inhibited. Contralateral acetone treatment on the non-desensitized left ear induced 74-91% ear swelling.

Treatment of TRPV1^{+/+} mice with ibuprofen (70 mg/kg) completely prevented ear swelling induced by contralateral acetone application. Ibuprofen treatment did not cause significant change in PMA-oedema on the right.

Effect of the lack of TRPV1 receptor and RTX pre-treatment on myeloperoxidase activity of ear samples. Contralateral acetone treatment of the left ears induced moderate, but significant increase in myeloperoxidase activity in TRPV1 knockout mice.

Strongly significant increase in MPO activity was observed in PMA-challenged right ears after 12 hours. Neither systemic RTX pre-treatment, nor deletion of TRPV1 receptors caused significant inhibition in MPO activity.

Histological findings. Highest number of microabscesses were counted in the ears of TRPV1 receptor knockout animals. Contralateral acetone treatment of the left ears was able to induce moderate microabscess formation in knockout animals. PMA treatment of the right ears led to a pronounced increase of mononuclear and polymorphonuclear cells both in TRPV1^{+/+}, TRPV1^{-/-} and RTX-pre-treated wild-type mice. Bilateral acetone smearing did not cause significant cell accumulation.

Effect of PMA and acetone treatment on local IL-1 β content of the mouse ear. Increased IL-1 β level was measured on the acetone treated left side in PMA-treated (right ears) animals. Bilateral acetone treatment did not cause IL-1 β elevation either in the ears of TRPV1^{+/+} or TRPV1^{-/-} animals. PMA treatment on the right side resulted in similarly elevated local IL-1 β levels in the ears of TRPV1^{+/+} and TRPV1^{-/-} mice.

Effect of systemic anti-IL-1 β antibody treatment on PMA- and acetone-induced ear oedema. Pre-treatment with anti-IL-1 β antibody did not ameliorate the potentiating effect of PMA on contralateral (left side) acetone-induced ear swelling. The antibody treatment also did not diminish PMA-induced right ear inflammation.

Discussion

We demonstrated the potentiating action of PMA on contralateral acetone-induced ear oedema and suppose the important role of prostanoids in this process. According to our data, IL-1 β is not essential in this case of sensitization. Our results prove that the potentiating effect is mediated via capsaicin-sensitive nerve fibres of the acetone-treated ear, but not via TRPV1 receptor. Furthermore we provided evidence that the neurogenic component of PMA-induced ear inflammation is independent of TRPV1 receptor and IL-1 β does not mediate this inflammatory process. Our data reveal that acetone cannot be used as an inert solvent of PMA in internally-controlled *in vivo* inflammatory animal studies. Our study sheds light on a systemic effect of non-neurogenic

mediators released from a local cellular inflammatory response which enhances neurogenic inflammation in distant parts of the body.

Role of capsaicin-sensitive afferents in dextran sulphate sodium-induced colonic inflammation

Involvement of capsaicin-sensitive nerve endings and TRPV1 receptor in inflammatory bowel disease has been investigated widely. The two most studied animal models of ulcerative colitis and Crohn's disease are oral dextran sulphate sodium (DSS)- and topical trinitrobenzene sulphonic acid (TNBS)-induced colonic inflammation, respectively (Okayasu et al., 1990; Morris et al., 1989).

Topical co-administration of 640 $\mu\text{mol/l}$ capsaicin inhibited TNBS-induced inflammation in rats (Goso et al., 1993) showing the anti-ulcerative properties of neuropeptides released by capsaicin. Orally administered capsaicin was also protective against oral dextran sulphate sodium-induced colitis in rats (Okayama et al., 2004). Some publications presented evidence that different TRPV1 receptor antagonists exert inhibitory action on TNBS- and DSS-evoked colitis in rodents (Kihara et al., 2003; Fujino et al., 2004; Kimball et al., 2004). Desensitization of sensory nerve endings by capsaicin pre-treatment exacerbated inflammatory changes in TNBS, DSS and various other models (Barada et al., 2001; Okayama et al., 2004), but data are also available that neonatal capsaicin desensitization attenuates DSS colitis in rats (Kihara et al., 2003).

Since experimental data concerning the role of capsaicin-sensitive sensory fibres and especially the TRPV1 receptor are strongly contradictory, the aim of the present study was to investigate the participation of sensory neurogenic components in DSS-induced colitis using TRPV1 receptor gene knockout mice and systemic RTX pre-treatment.

Methods

Induction of colitis. DSS (2%) dissolved in millipore water was administered orally to mice *ad libitum* for 6 or 5 days. Control animals received millipore water. Animals were weighed, stool consistency, and blood content were scored daily. After 6 days animals were anaesthetized in saturated diethyl aether vapour and killed by decapitation. Colons were removed and gently flushed with Krebs-Henseleit solution to remove faeces. Colons were cut into three equal segments (proximal, intermediate and distal). Four mm wide rings were obtained from each colon segment for histological examination.

Systemic resiniferatoxin pre-treatment. Systemic RTX pre-treatment was performed and validated as described previously.

Disease activity index. Weight loss, stool consistency and blood content of stool were scored daily as described previously (Stevceva et al., 2001). Each parameter was given a score ranging from 0 to 4. Disease activity index is the mean of weight loss, stool consistency and stool blood content scores. Survival analysis of TRPV1^{+/+}, TRPV1^{-/-} and TRPV1^{+/+} RTX pre-treated DSS drinking animals was also performed.

Myeloperoxidase assay. Colon segments were weighed, chopped into small pieces and homogenized in 1 ml of 50 mM potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (pH 6). The homogenate was centrifuged at 10000 g, 4 °C for 10 minutes. Samples of the supernatant were collected (400 µl). Myeloperoxidase activity was determined as described above.

Histology. Crypt height, severity and extent of inflammation were scored as described earlier (Kihara et al., 2003).

Results

Disease activity index. Two percent DSS solution led to death of 57.14% of TRPV1^{+/+} animals. Genetic lack of TRPV1 receptor did not affect disease activity index. Loss of TRPV1^{-/-} mice was 38.46% in 6 days. At the RTX pre-treated TRPV1^{+/+} animal group we experienced enormous loss of animals (91.67%). Systemic RTX pre-treatment did not change disease activity index values. Survival curve of TRPV1^{+/+} RTX-pre-treated DSS drinking animals showed significant lower survival probability.

Myeloperoxidase activity of colon samples. DSS treatment induced significant elevation of MPO level in all experimental groups. TRPV1 knockout animals showed more than two-fold increase of MPO activity in the intermediate colon segment. In intermediate and distal colon segments functional block of capsaicin-sensitive afferent fibres by systemic RTX pre-treatment led to significantly higher MPO activity values.

Histology. Composite histological scores of dextran sulphate sodium-treated animals were significantly higher than those of water-treated ones in all colon segments and all animal groups. Genetic lack of the TRPV1 receptor induced significantly more enhanced histological changes in the intermediate and distal colon segments. RTX pre-treatment resulted in significantly more pronounced inflammatory processes.

Discussion

Animal models of experimental ulcerative colitis are useful tools for investigating the pathomechanism of the disease. Dextran sulphate sodium-induced colitis shows classical symptoms of ulcerative colitis which are chronic relapsing diarrhoea, rectal bleeding and inflammation, mucosal ulceration and microscopic crypt abscesses. Ulcerated areas demonstrate markedly increased epithelial cell apoptosis and necrosis on histologic examination (Boismenu et al., 2002; Vetuschi et al., 2002).

Data about involvement of TRPV1 receptor and sensory neuropeptides in DSS colitis are contradictory. Kihara and Kimball found that various neuropeptides liberated from capsaicin-sensitive nerve endings have pro-inflammatory cumulative effect. In contrast of these findings, Okayama and co-workers demonstrated that orally administered selective TRPV1 receptor agonist capsaicin, which releases sensory neuropeptides, inhibited DSS-induced colitis in rats. They also established that capsaicin desensitization increased the inflammatory response (Okayama et al., 2004). Their results suggest anti-inflammatory cumulative effect of sensory neuropeptides in this model. Our findings are in keeping with those of Okayama and underline that desensitization of the capsaicin-sensitive sensory nerves by RTX or genetic deletion of TRPV1 receptor exacerbates the histopathological changes and decreases the survival probability in the DSS colitis.

In conclusion, to learn more about modulatory role of TRPV1 receptors and capsaicin-sensitive nerve endings may contribute to the development of novel type anti-inflammatory drugs available in the therapy of ulcerative colitis.

Novel findings

1. Our data demonstrate that PACAP-38 can be released from the sensory nerves of the rat trachea either by capsaicin treatment or electrical field stimulation. We also provide evidence that PACAP-38-like immunoreactivity can be detected in the naïve rat plasma. The presence of PACAP-38 in the rat plasma was verified by mass spectrometry. Resiniferatoxin proved to be able to induce a two-fold increase in the plasma PACAP-38 level.
2. Our studies evince anti-inflammatory action of PACAP-38 against both neurogenic and mixed type inflammatory stimuli. PACAP-38 dose dependently inhibited capsaicin and electrical field stimulation-evoked neuropeptide release in the rat trachea. Systemic applied PACAP-38 significantly diminished mustard oil-induced neurogenic plasma extravasation in the mouse ear, mustard oil-induced inflammation in the rat hindpaw and neurogenic inflammation of the rat hindpaw evoked by intraplantar capsaicin or resiniferatoxin.

- Intravenous PACAP-38 successfully mitigated carrageenan-induced inflammation of the rat hindpaw.
3. We observed sensitizing effect of PMA-induced inflammation on acetone-evoked oedema in the mouse ear. Capsaicin-sensitive sensory nerve endings play a pivotal role in the mechanism of potentiation, because systemic resiniferatoxin pre-treatment inhibited the sensitizing effect. Using genetically modified animals we also proved that TRPV1 receptor is not essential in this phenomenon. We found that prostanoids are important mediators in the process, but IL-1 β does not have any important action.
 4. Our results elucidated some unclear details of the pathomechanism of PMA induced inflammation. We found that genetic lack of TRPV1 receptor did not influence the condition. Our data show that IL1- β is not essential in mediating PMA evoked ear inflammation.
 5. Our studies demonstrated that functional ablation of capsaicin-sensitive afferent nerve endings and genetic lack of TRPV1 receptor exacerbate inflammatory cell accumulation and other histological features of dextran sulphate sodium-induced colitis in C57BL/6 mice.

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Papers related to the thesis

- Németh J, Reglódi D, Pozsgai G, et al. Effect of pituitary adenylate cyclase activating polypeptide-38 on sensory neuropeptide release and neurogenic inflammation in rats and mice. *Neuroscience* 2006;143:223-230. IF: 3.427
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