Role of neurogenic components in inflammatory diseases of the skin, joint and colon

PhD thesis

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Contents

Abbreviations

ANKTM1	Ankyrin-like protein with transmembrane domains 1
ANOVA	analysis of variance
ATP	adenosine triphosphate
AUC	area under the curve
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CGRP	calcitonin gene-related peptide
CRLR	calcitonin receptor-like receptor
DSS	dextran sulphate sodium
EC_{50}	half maximal effective concentration
EDTA	ethylenediamine tetraacetic acid
EFS	electrical field stimulation
ELISA	enzyme-linked immunosorbent assay
IgG	immunoglobuline G
IL-1β	interleukin-1 beta
LPS	lipopolysaccharide
MALDI TOF	matrix-assisted laser desorption/ionization time of flight mass spectrometry
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MW	molecular weight
NK1, 2, 3	neurokinin 1, 2, 3 receptors
NKA, B	neurokinin A, B
NO	nitric oxide
OD	optical density
PAC1	PACAP type I receptor
PACAP	pituitary adenylate cyclase activating polypeptide
PACAP-LI	PACAP-like immunoreactivity
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate

PPT-A, B	preprotachykinin A, B
RAMP-1	receptor-activity-modifying protein 1
RIA	radioimmunoassay
RTX	resiniferatoxin
SEM	standard error of the mean
SP	substance P
TNBS	trinitrobenzene sulphonic acid
TRPA1	transient receptor potential channel A1
TRPV1	transient receptor potential vanilloid 1 receptor
VPAC1, 2	VIP PACAP receptor 1, 2
VR1	vanilloid receptor subtype 1

Introduction

Capsaicin-sensitive nociceptive afferent neurons

Nociceptive afferent neurons transport information into the central nervous system about potentially noxious stimuli. Somata of nociceptive primary afferent neurons innervating the skin are located in dorsal root ganglia and trigeminal ganglia. Cell bodies of extrinsic afferent neurons innervating the gastrointestinal tract lie in the jugular and nodose ganglia or in the dorsal root ganglia. They reach the central nervous system via the vagal and spinal nerves (Grundy, 2002; Holzer, 2002). Axons of these neurons are either myelinated (Aδ) or unmyelinated (C).

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; Koltzenburg & McMahon, 1986) leads to vasodilatation (Bayliss, 1923; Langley, 1923) and subsequent plasma extravasation. This phenomenon is called neurogenic inflammation, because it is prevented by desensitization of sensory nerves or denervation (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 the pungent ingredient of hot chilli pepper, 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. Tachykinins (substance P, neurokoinin A and B) are responsible for plasma extravasation, mast cell degranulation and leukocyte accumulation. Calcitonin gene-related peptide (CGRP) induces vasodilatation (Holzer, 1991; Maggi, 1995; Brain 1997). Capsaicin-sensitive afferents also release neuropeptides associated with anti-inflammatory actions (somatostatin, galanin, PACAP-38), some of them can be detected in the systemic circulation (Szolcsányi et al., 1998a). Besides inflammatory actions sensory neuropeptides influence smooth muscles of the gastrointestinal tract (Barthó et al., 2004). This means that capsaicin-sensitive sensory neurons exert triple function: afferent, local efferent (pro-inflammatory) and systemic efferent (anti-inflammatory) effects.

Besides antidromic electrical stimulation capsaicin-sensitive neurons can be activated orthodromically by noxious heat, mechanical and chemical stimuli. Chemical substances include pungent agents (capsaicin, resiniferatoxin and mustard oil) or protons. Capsaicin is the pungent ingredient of red pepper (Capsicum genus). It has vanilloid structure (8-Methyl-N-vanillyl-trans-6-nonenamide) and activates sensory fibres via transient receptor potential vanilloid 1 (TRPV1) receptors. Repeated administration of high doses desensitizes and induces destruction of terminal parts of a major subpopulation of nociceptive sensory nerve fibres. Multiple application of capsaicin depletes the peptide content of sensory nerve endings (Jancsó & Jancsó-Gábor, 1959; Jancsó, 1960; Szolcsányi, 1977; Szolcsányi, 1996). Resiniferatoxin (RTX) is the irritant principle of Euphorbia resinifera. It is also a vanilloid compound and related to phorbol substances. It activates protein kinase C (PKC), but is not tumorigenic. RTX is an ultrapotent capsaicin analogue, which is more suitable for desensitization of capsaicin-sensitive nerve fibres because of less unwanted effects (Szállási & Blumberg, 1989; Szállási et al., 1990; Szolcsányi et al., 1990; Szállási & Blumberg, 1999). Plants of the Brassica genus contain mustard oil (allyl isothiocyanate). When applied at a concentration below 20% (according to other authors below 5%) allyl isothiocyanate selectively excites capsaicin-sensitive afferents in rodent skin. Unlike capsaicin, mustard oil does not cause desensitization of nerve terminals, so treatment can be performed repeatedly (Jancsó et al., 1967; Jancsó et al., 1977; Inoue et al., 1997; Bester et al., 1998; Laird et al., 2000). Mustard oil activates TRPA1 (former ANKTM1) receptors, which, like TRPV1 capsaicin receptors, are members of transient receptor potential (TRP) receptor superfamily (Jordt et al., 2004). 1% mustard oil causes neurogenic inflammation without the involvement of mast cells (Inoue et al., 1997; Szolcsányi et al., 1998a).

Neurogenic inflammation may contribute to the pathomechanism of several diseases like bronchial asthma (Barnes, 1986; Bertrand et al., 1993; Germonpré et al., 1995; 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

The existence of a capsaicin receptor molecule in the cell membrane of a subpopulation of nociceptive sensory neurons was put forward long ago (Szolcsányi & Jancsó-Gábor, 1975). Recently, the cloning of capsaicin receptor succeeded (Caterina et al., 1997; Caterina & Julius, 2001; Clapham et al., 2003). Initially the receptor was called vanilloid receptor 1 (VR1). Later homology was found with transient receptor potential (TRP) ion channels and the name was modified for transient receptor potential vanilloid 1 (TRPV1) (Gunthorpe et al., 2002).

TRPV1 proteins contain 6 transmembrane domains and four protein subunits form a functional non-selective cation channel (Caterina et al., 1997; Caterina et al., 1999; Smith et al., 2002; Watanabe et al., 2002). The cation channel 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; Blumberg et al., 1993; Liu & Simon, 1996; Caterina et al., 1997; Szállási & Blumberg, 1999; Liu et al., 2000; Dedov et al., 2002), endogenous compounds (anandamide, lipoxygenase products, N-oleoyl-dopamine) (Hwang et al., 2000; Benham et al., 2002; DiMarzo et al., 2002; Szolcsányi, 2002; Trevisani et al., 2002; Chu et al., 2003). TRPV1 receptor can be considered an integrator nociceptive transducer membrane protein suitable for signalling of noxious chemical and heat stimuli in the peripheral endings of polymodal nociceptors (Tominaga et al., 2001; Garcia-Martinez et al., 2002; Szolcsányi & Pethő, 2006).

Opening of the non-selective TRPV1 ion channel leads to inwardly rectifying Ca^{2+} and Na^{+} currents. These currents depolarize the neuronal membrane and result in action potential formation. Rise of intracellular Ca^{2+} concentration also plays a role in the exocytosis of neuropeptides. Elevated intracellular Ca^{2+} level switches on several signal transduction pathways that contribute to the desensitization of the afferent neuron (Koplas et al., 1997; Liu & Simon, 1998).

Phosphorylation of TRPV1 protein by various enzymes induces sensitization or activation. One of these enzymes is protein kinase C (PKC) (Cesare et al., 1999; Chuang et al., 2001; Varga et al., 2006). PKC is a phospholipid-dependent enzyme, which plays pivotal role in several signal transduction processes. Activation of PKC is implicated in the pathogenesis of inflammation via phospholipase A_2-dependent

arachidonic acid release and eicosanoid production (Silvan et al., 1996; Bermejo et al., 1998). Recent papers have been revealed that PKC activation by phorbol 12-myristate 13-acetate (PMA) elicited nocifensive behaviours exclusively via sensitization/activation of TRPV1 receptors, since the PMA-induced nocifensive reaction was completely absent in TRPV1 knockout animals (Bölcskei et al., 2005) or TRPV1 receptor antagonists strongly inhibited it (Ferreira et al., 2005).

Pro-inflammatory sensory neuropeptides

Main members of pro-inflammatory sensory neuropeptides include tachykinins (substance P, neurokinin A, neurokinin B) and CGRP. The tachykinins hemokinin-1 and endokinins were discovered recently.

Tachykinins share a common C terminal amino acid sequence. Substance P (SP) was isolated by von Euler and Gaddum from the brain and intestine of the horse in 1931. Main sources of tachykinins are capsaicin-sensitive sensory neurons, but they can also be found in some non-neurogenic elements. Tachykinins were evinced in capsaicin-resistant large neurons with Aβ axons following inflammation-induced plasticity (Neumann et al., 1996), in capsaicin-resistant neurons of the airways (Hunter et al., 2000), and in the enteric nervous system (Holzer & Holzer-Petsche, 1997; Lomax & Furness, 2000). SP was also detected in endothelial, inflammatory and immune cells (Aliakbari et al., 1987; Linnik & Moskowitz, 1989; Pascual & Bost, 1990; Ho et al., 1997; Maggi, 1997; Lai et al., 1998). SP and neurokinin A (NKA) are translated from preprotachykinin A gene (PPT-A), whereas neurokinin B (NKB) is translated from preprotachykinin B gene (PPT-B). Tachykinins activate G protein coupled receptors (NK1, 2, 3). NK1 receptor is present both in the central and peripheral nervous systems. It was also detected on endothelial and smooth muscle cells (Patacchini & Maggi, 2001). Inducible form of NK1 receptor is expressed in bone marrow cells (Bandari et al., 2003). NK2 receptor is mostly located in the periphery, but it can be found in some nuclei of the central nervous system. NK3 receptor is mainly present in the central nervous system and in some peripheral organs, such as the uterus, striated muscles, lungs, the liver, portal and mesenteric veins, enteric neurons (Tsuchida et al., 1990; Massi et al., 2000). 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. The recently discovered hemokinin-1, endokinin A and B are also full agonists of NK1, 2 and 3 receptors. Endokinin A and B may be the endogenous ligands of NK1 receptors in the non-innervated areas (Page et al., 2003).

Tachykinins mediate diverse effects. 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 via NK1 receptor. They facilitate proliferation, migration of endothelial cells and angiogenesis (Maggi, 1995). In some vascular areas tachykinins cause vasoconstriction via NK1, 2 and 3 receptors. Tachykinins evoke plasma protein extravasation in both somatic and visceral areas by activating NK1 receptors. Smooth muscle contraction can be produced by tachykinins indirectly, via release of other mediators and mast cell degranulation. They can also contract smooth muscles directly. NK1 and 2 receptors were evinced in the smooth muscles of several organs (Maggi, 1991, 1995). Mast cell degranulation by SP was thought to be evoked by basic amino acids of the N terminal (Maggi, 1997). Recently, NK1 receptors were found in mast cells (Cao et al., 1999). SP stimulates the secretion of salivary, intestinal glands and the pancreas (Lembeck & Starke, 1968; Konturek et al., 1981).

CGRP was discovered by Amara and co-workers (Amara et al., 1982). It exists in two variant forms (αCGRP and βCGRP) which are produced by alternative splicing. CGRP belongs to the calcitonin/CGRP peptide family, other members of which are islet amyloid polypeptide and adrenomedullin. CGRP acts via G protein coupled receptors. CGRP receptor consists of calcitonin receptor-like receptor (CRLR) and receptor-activity-modifying protein 1 (RAMP-1) (Njuki et al., 1993; McLatchie et al., 1998).

CGRP has mostly extraneural effects. It facilitates the release of acethylcholine 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 and represents direct action on smooth muscle cells (Edvinsson et al., 1985; Han et al., 1990; Hughes & Brain, 1994). However, dilation of the aorta is endothelium-dependent and is mediated via nitric oxide (NO) (Brain et al., 1985; Gray & Marshall, 1992). Another possible mechanism of CGRP-evoked vasodilation is activation of ATP-sensitive potassium channels by the protein kinase

A (PKA) pathway (Nelson et al., 1990; Quayle et al., 1994; Brain & Grant, 2004). Vasodilatatory effect of CGRP can be detected mainly on arteries and arterioles. CGRP itself does not enhance vascular permeability, but potentiates the action of SP (Cao et al., 2000). Similarly to the vasodilatatory effect, CGRP induces smooth muscle relaxation in several organs (Maggi, 1995). CGRP exerts positive chronotropic and inotropic actions on the heart due to elevation of cAMP level (Tippins et al., 1984). Under *in vitro* conditions CGRP facilitated attachment of neutrophils to vascular endothelium by raising the expression of adhesion molecules (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

Sensory ganglia contain several peptides, but not all of them have real functional significance in sensory-mediated processes (Hökfelt et al., 1976; Maggi, 1995). Pituitary adenylate cyclase activating polypeptide (PACAP) is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family and was originally isolated from ovine hypothalamus (Miyata et al., 1989). PACAP exists in two forms (PACAP-27 and PACAP-38). The 38 amino acid form is the predominant in mammalian tissues.

Two types of PACAP binding sites were evidenced in different tissues. Type I binding sites have high affinity for PACAP-38 and 27, but low affinity for VIP (Suda et al., 1992). Type II binding sites have similar affinity for PACAP and VIP (Gottschall et al., 1990; Lam et al., 1990). Type I binding sites represent PAC1 receptors which are specific for PACAP (Pisegna & Wank, 1993). Type II biding sites represent VPAC1 and 2 receptors which bind PACAP and VIP, too (Ishihara et al., 1992; Lutz et al., 1993; Arimura, 1998; Sherwood et al., 2000; Vaudry et al., 2000). The receptors of PACAP are G protein-coupled receptors. PACAP receptors are widely distributed throughout the central nervous system and peripheral organs.

PAC1 receptor is the predominant form in the brain. Its mRNA was detected in the olfactory bulb, the dentate gyrus of the hippocampus, the supraoptic nuclei of the hypothalamus, the cerebellar cortex and area postrema (Hahsimoto et al., 1996; Nomura et al., 1996; Shioda et al., 1997; Otto et al., 1999). 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). VPAC1 and 2 mRNAs overlap only in the hippocampus (Usdin et al., 1994). PACAP receptors are located both on neurons and glial cells (Tatsuno et al., 1991; Grimaldi & Cavallaro, 1999). Messenger RNAs of PACAP binding receptors were detected in most endocrine glands including the pituitary, the pancreas, the adrenal gland and gonads (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 peripherwal tissues (Somogyvári-Vigh & Reglődi, 2004; Zhou et al., 2002). PACAP is reported to regulate cell division, cell cycle arrest, differentiation and cell death. It seems to have a protective function in neurons, but it promotes apoptosis in tumor cells (Spengler et al., 1997; Gillardon et al., 1998). PACAP plays an important role in the development of the brain, the liver, the adrenal gland and the pancreas (Le Meuth et al., 1991; El Fahime et al., 1996; DiCicco-Bloom et al., 1998; Yon et al., 1998). PACAP and its various receptor forms are found in many cancers. They are involved in both proliferation and differentiation. PACAP exerts primariliy relaxant effect on blood vessels. Although, fall of blood pressure induced by intravenous administration of PACAP is followed by an increase in cats and dogs (Ishizuka et al., 1992). The secondary vasoconstriction may result from sympathetic regulation, differential receptor use, PACAP-induced arginine vasopressin or catecholamine release. PACAP evokes smooth muscle relaxation in the respiratory, digestive and reproductive organs. It has positive inotropic, chronotropic and dromotropic effects in the heart. PACAP participates in immune cell protection, phagocytosis and

immune response. It plays a role in bone formation (Strange-Vognsen et al., 1997), resorption (Winding et al., 1997) and haematopoiesis (Cai et al., 1997). PACAP regulates endocrine functions of the hypothalamus, the pituitary, gonads, the adrenal gland, the pancreas and enterochromaffin-like cells of the gastrointestinal tract. It influences exocrine secretion of the stomach, the pancreas and seromucous glands (Cardell et al., 1991; Raufman et al., 1991; Felley et al., 1992; Mungan et al., 1995; Onaga et al., 1996). PACAP stimulates melatonin synthesis in the pineal gland (Simonneaux et al., 1998). The level of PACAP in retinal ganglion cells is low in the day and high at night (Fukuhara et al., 1997). PACAP takes part in the regulation of autonomic functions associated with the medulla oblongata, otic ganglia, sphenopalatine ganglia and the jugular nodose ganglia (Legradi et al., 1994; Mulder et al., 1995). Presence of PACAP in ciliary ganglion neurons, jugular and nodose ganglia, dorsal horn neurons, spinal cord, mesencephalic trigeminal nucleus and the vagal nerve indicate its role in sensory neurotransmission (Mulder et al., 1995; Zhang et al., 1995; Dickinson et al., 1997; Larsen et al., 1997; Zhang et al., 1997; Reimer et al., 1999). PACAP may have a stress-related behavioural function (Kozicz et al., 1997). It is involved in behaviour associated with increased grooming, pain responses, reduced food intake, motor activity and body temperature (Morley et al., 1992; Masuno et al., 1995; Narita et al., 1996; Zhang et al., 1996; Masuno et al., 1997; Mizuno et al., 1998). It enhances rapid eye movement sleep. This is partly due to facilitation of prolactin secretion (Bredlow et al., 1994; Fang et al., 1995).

As mentioned above, presence of PACAP-like immunoreactivity was evidenced in the superficial dorsal spinal horn layers and in small-sized neurons of the dorsal root ganglia (Moller et al., 1993; Mulder et al., 1994; Dun et al., 1996). PACAP was also detected in a population of capsaicin-sensitive sensory neurons (Zhang et al., 1996). In sensory ganglia, nerve plexus of inner organs (Fahrenkrug & Hannibal, 1998; Hannibal et al., 1998; Skakkebaek et al., 1999; Hannibal & Fahrenkrug, 2000; Schoenfeld et al., 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; Schoenfeld et al., 2000; Mirabella et al., 2001).

Aims

We planned to investigate the role of capsaicin-sensitive afferent nerve terminals and TRPV1 receptors in different animal models of inflammation. In particular, we were interested in sensory neuropeptides that may have systemic anti-inflammatory properties. We performed both *in vitro* and *in vivo* experiments using systemic desensitization of capsaicin-sensitive nerves and TRPV1 receptor gene knockout animals. Our goals were the following:

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-1β 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

Introduction

Numerous studies suggest that PACAP has immunomodulatory actions, which together with those of VIP are mainly anti-inflammatory (Delgado et al., 1999a; Abad et al., 2001; Abad et al., 2006; 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. PACAP is produced by B and T lymphocytes, but not by peritoneal macrophages (Gaytan et al., 1994; Abad et al., 2002). Expression of PACAP receptors has been demonstrated in most immune cell types. B and T cells express VPAC1 and 2 receptors. Macrophages express PAC1, VPAC1 and 2 receptors (Pozo et al., 1997; Qian et al., 2001). VPAC2 is inducible in T cells and macrophages (Delgado et al., 1999b). Most important anti-inflammatory effect of PACAP is direct inhibition of macrophage activity (Delgado et al., 1999b). It also stimulates the secretion of anti-inflammatory cytokines (Delgado et al., 1999c) and down-regulates the expression of the macrophage co-stimulatory molecules B7.1 and B7.2 (Greenwald et al., 2005). PACAP may stimulate or inhibit the secretion of immunoglobulines depending on the source of B lymphocytes and the immunoglobuline isotype (Delgado et al., 2004). It also diminishes neutrophil chemotaxis (Kinhult et al., 2001). PACAP promotes Th2 differentiation above Th1 responses. This involves impeding signalling molecules that facilitate Th1 reaction (IL-12) and selective generation of Th2 memory cells (Delgado et al., 2000; Delgado et al., 2002).

These data prognosticate a beneficial effect of PACAP in inflammatory diseases characterized by macrophage overactivity and an overbalanced Th1/Th2 response. Animal models of inflammation in which PACAP was successfully applied include

LPS-induced septic shock, asthma models, collagene-induced arthritis, experimental autoimmune encephalomyelitis and brain injury (Saotome et al., 1998; Delgado et al., 1999d; Abad et al., 2001; Kato et al., 2004).

Because lack of data about actions of PACAP in neurogenic inflammatory processes, we focused our experiments on this field.

Methods

Animals

Experiments were carried out on male Wistar rats and Balb/c mice. Animals were bred in the Animal House of Pécs University under pathogen free condition at 24-25 ^{o}C in climatically controlled environment, given standard diet and water *ad libitum*.

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

Rats were exsanguinated in deep anaesthesia (thiopental sodium, 50 mg/kg i.p.). The whole trachea was removed. Tracheae from two rats were placed into the same organ bath (1.8 ml) and perfused (1 ml/min) with pH 7.2 oxygenized Krebs solution for 60 min at 37 ^{o}C. After the equilibration period pre-stimulated, stimulated and post-stimulated fractions were collected, 8 min each. In the second 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.

PACAP-38, CGRP, substance P and somatostatin concentrations were determined from the incubation medium by radioimmunoassay (RIA) (Németh et al., 1996; Helyes et al., 1997; Németh et al., 1998; Németh et al., 1999; Jakab et al., 2004). For the specific RIA assays the antisera (PACAP-38: "88111-3"; SP: "L83"; CGRP: "C1012"; somatostatin: "775/7") were raised in rabbits or, in case of somatostatin, in sheep immunised with synthetic peptides conjugated to bovine serum albumin or thyroglobulin by glutaraldehyde or carbodiimide. 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 μl, 1:10000), the RIA tracer (100 μl, 5000 cpm/tube) and the standard or unknown samples (100 μl) were put into the assay buffer. After 48-72 h incubation at 4 °C, the antibody-bound peptide was separated by adding 100 μl separating solution (10 g

charcoal, 1 g dextran and 0.5 g fat-free milk powder in 100 ml distilled water). Following centrifugation (3000 rpm, 4 °C, 15 min) the radioactivity of the precipitates was measured. Peptide concentrations of the unknown samples were calculated from a calibration curve.
To examine the effect of PACAP-38 on the release of the sensory neuropeptides it was added into the incubation medium at the beginning of each 8 min fraction in concentrations of 20, 100, 500 and 2000 nM.

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 to demonstrate that the plasma samples contain PACAP-38 and do not contain PACAP-27. The aqueous solutions of the PACAP-38 standard and the naïve rat serum sample were loaded onto the target plate by mixing 1 µl of each solution 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. Each spectrum was detected in linear mode. The instrument uses a 337 nm pulsed nitrogen laser. External calibration was performed in each case using Bruker Peptide Calibration Standard. Protein masses were acquired with a range of 1000 to 8000 m/z. Each spectrum was produced by accumulating data from 300 consecutive laser shots.

Systemic stimulation of capsaicin-sensitive afferents *in vivo*

Tail veins of anaesthetized rats (thiopental sodium, 100 mg/kg, i.p.) were cannulated and resiniferatoxin, an ultra-potent TRPV1 receptor agonist was administered (3 µg/kg, i.v.) (Szállási & Blumberg, 1989; Helyes et al., 2003). Tracheal T-cannula was inserted for artificial respiration when required. Blood samples were taken by cardiac puncture 5 min after RTX injection.

Antidromic stimulation of the sciatic nerve

Electrical stimulation of peripheral stumps of cut sciatic nerves of anaesthetized rats (thiopental sodium, 100 mg/kg, i.p.) was performed (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 to counteract the vascular effects of sympathetic nerve activation. Pipecuronium bromide (200 μg/kg, i.v.) was injected to block neuromuscular transmission and positive pressure ventilation was carried out through a T tracheal tube with room air. Tidal volume was set for 10 ml/kg and respiratory rate was adjusted to 100 cycles/min. Blood samples were taken by cardiac puncture 5 min after the end of the stimulation period.

Plasma preparation for radioimmunoassay

Plasma concentration of PACAP-38 was determined by RIA (Jakab et al., 2004). Blood samples (6 ml per animal) were taken by cardiac puncture and mixed with EDTA (12 mg) and aprotinin (1200 U). Following centrifugation (2000 rpm for 10 min at 4 °C) the peptide was extracted from the plasma by addition of 3 volumes of absolute alcohol. After second centrifugation (2000 rpm for 10 min at 4 °C) the samples were dried under nitrogen flow and resuspended in 300 μl assay buffer (Jakab et al., 2004). RIA was performed as described above.

Measurement of mustard oil-induced neurogenic oedema formation in the mouse ear

Male Balb/c mice (20-25 g) were anaesthetized with ketamine (100 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.). Neurogenic 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) before the treatment and 4 times during the 3 h examination period. Oedema was expressed in %

compared to the initial control values. PACAP-38 (10, 100 and 1000 μg/kg in 200 μl saline, i.p.) was administered 15 min before both mustard oil smearing. Animals of the control group were treated with the same volume of saline. The assigned PACAP dose was based on earlier studies (Abad et al., 2001; Kato et al., 2004). Area under the curve (AUC) values were calculated (arbitrary units are mm x min) and analyzed. At the end of the experiment mice were sacrificed by anaesthetic overdose, the ears were dissected and histology was performed. Oedema formation in the connective tissue was evaluated.

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

Both hindlegs of male Wistar rats weighing 180-250 g were acutely denervated (the sciatic and the saphenous nerves were cut 30 min before the induction of inflammation) under pentobarbital sodium (40 mg/kg, i.p.) anaesthesia to avoid 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 i.v. and neurogenic inflammation was induced 10 min later. Rats were killed by exsanguination 20 min after mustard oil application. The skin of the hindpaws was removed and the extravasated dye was extracted with formamide. Dye concentration was determined by spectrophotometry at 620 nm and was expressed as μg dye/g wet tissue (Helyes et al., 1997; Helyes et al., 2001). PACAP-38 (100 μg/kg, i.p.) was administered 20 min before the induction of inflammation. This systemic dose was chosen on the basis of the results of the mouse ear neurogenic oedema studies.

Measurement of acute neurogenic inflammation evoked by TRPV1 receptor agonists in the plantar skin of the rat hindpaw

Both hindlegs of the anaesthetized (pentobarbital sodium, 40 mg/kg, i.p.) rats were acutely denervated as described abobe. Acute neurogenic inflammation was evoked

by intraplantar injection of RTX (100 μl, 0.1 μg/ml) or in another group capsaicin (100 μl, 100 μg/ml) into the left hindpaw. Extravasation of plasma albumin was measured by the Evans blue leakage method, as described above. Rats were exsanguinated 20 min after the application of RTX or capsaicin. The plantar skin of the hindpaws was removed and the extravasated dye was extracted, dye concentration was measured and expressed as μg dye/g wet tissue. Dye content of the skin of the right paw was taken off the values. PACAP-38 (10 μg/kg) or in the control group isotonic saline were administered i.p. 10 min before the induction of the 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). Oedema was expressed as % of initial values. 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). Animals of the control group received saline.

Statistics

Results are expressed as mean ± standard error of mean (SEM). For analyzing data Student's t-test and ANOVA followed by Bonferroni's or Dunnett's test were used. Probability values $p<0.05$ were accepted as significant.

Ethics

All experimental procedures were carried out according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Forbearance and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988) and complied

with the recommendations of the Helsinki Declaration. The studies were approved by the Ethics Committee on Animal Research of Pécs University according to the Ethical Codex of Animal Experiments.

Results

PACAP-38 is released from sensory nerve endings of the isolated rat trachea

Capsaicin (10^{-6} M) and electrical field stimulation (EFS) with C-fibre strength evoked 27% and more than two-fold elevation of PACAP-38 release, compared to the pre-stimulated basal values, respectively. Post-stimulation levels returned to the basal concentrations (Fig. 1).

PACAP-38, but not PACAP-27 can be detected in the plasma with mass spectrometry

PACAP-38 could be clearly 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) which is identical to PACAP-38 H^+ adduct (MW: 4535.03) found in the standard. The other biologically active form, PACAP-27 (MW: 3147.6) or its $[M+Na]^+$could not be detected (Fig. 3).

PACAP-like immonoreactivity of the rat plasma increases in response to systemic stimulation of capsaicin-sensitive nerves

Stimulation of capsaicin-sensitive nerves throughout the body by injection of the TRPV1 receptor agonist RTX (3 μg/kg i.v.) induced a 2-fold elevation of plasma PACAP-like immunoreactivity (PACAP-LI) compared to solvent-treated rats. Antidromic electrical excitation of the peripheral stump of the cut sciatic nerve slightly elevated PACAP-LI, but this local stimulation of cutaneous sensory fibres was not sufficient to significantly increase PACAP-LI in the systemic circulation (Fig. 2).

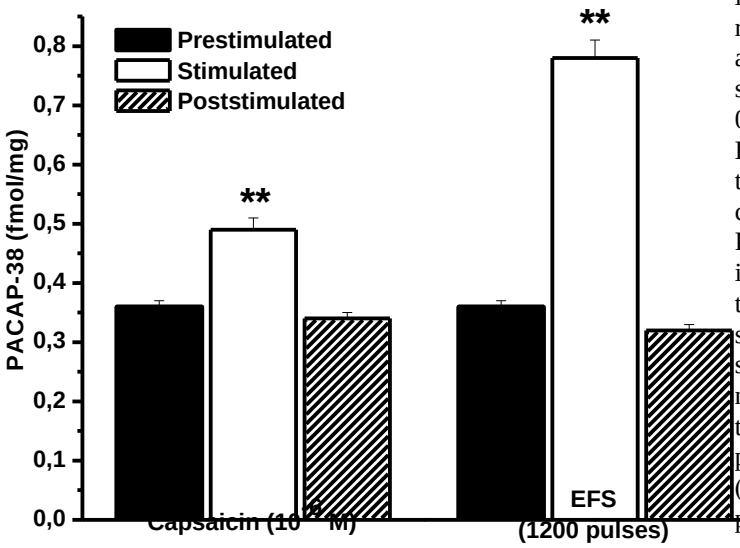

Figure 1. Release of PACAP-38 from isolated rat tracheae in response to capsaicin- and electrical field stimulation (EFS; 40 V, 0.1 ms, 10 Hz, 2 min). Each column represents the mean±SEM concentration of PACAP measured in the incubation medium of the pre-stimulated, stimulated and post-stimulated fractions of n=6 experiments (6x2 tracheae). **P<0.01 vs. pre-stimulated fractions (Student's t-test for paired comparison).

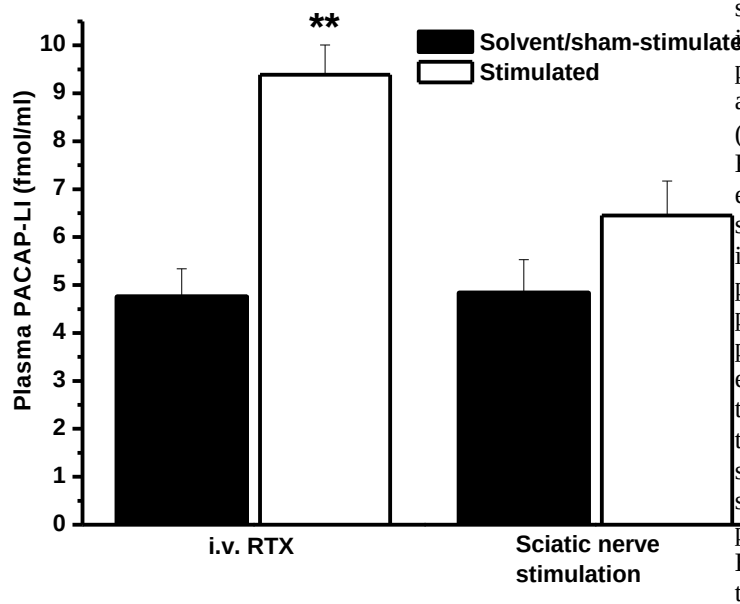

Figure 2. PACAP-like immunoreactivity (PACAP-LI) in the rat plasma. Plasma PACAP-LI significantly elevated 5 minutes after systemic stimulation of capsaicin-sensitive sensory nerves by i.v. injection of the ultra-potent TRPV1 receptor agonist resiniferatoxin (RTX; 3 μg/kg). However, local excitation of capsaicin-sensitive afferents innervating predominantly the plantar regions of the paw (antidromic electrical stimulation of the peripheral stump of the sciatic nerve) slightly, but not significantly increased plasma PACAP-LI. Each column represents the mean±SEM PACAP-LI of n=6 rats. **P<0.01 vs. stimulated (Student's t-test for unpaired comparison).

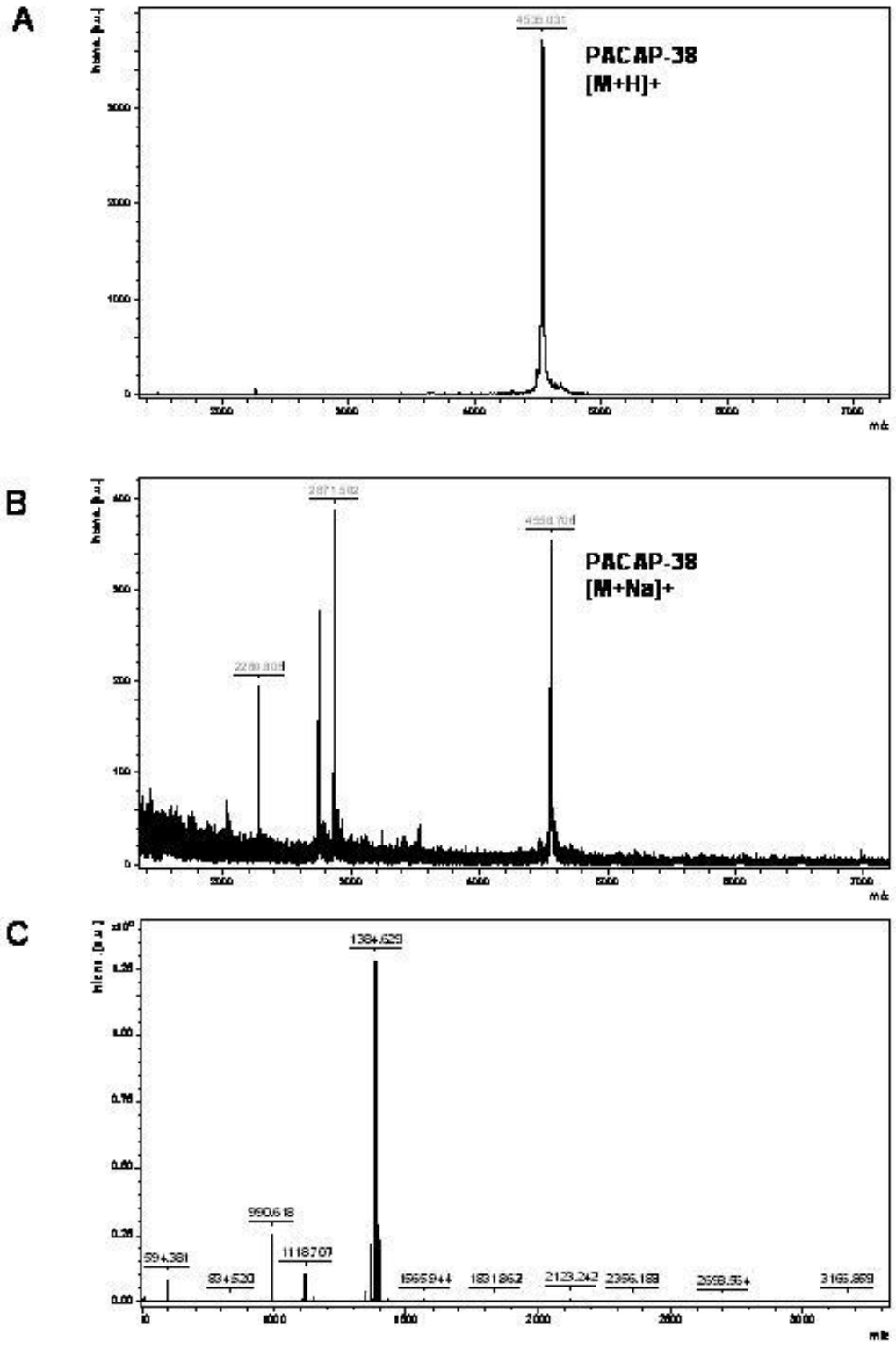

Figure 3. (**a**) Identification of PACAP-38 in the standard and (**b**) in plasma samples of naïve rats with mass spectrometry at the molecular weight signal of 4558.7 Da representing the quasimolecular ion of PACAP-38 Na^+ adduct (MW: 4558.7) which is identical to PACAP-38 H^+ adduct (MW: 4535.03) found in the standard. The other biologically active form, PACAP-27 (MW: 3147.6) or its $[M+Na]^+$ could not be detected. (**c**) Mass spectrum obtained with MALDI TOF/TOF measurement after on-plate tryptic digestion of PACAP-38 in the plasma samples.

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. Similarly, 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 measured sensory neuropeptides in both cases. However, it did not influence basal peptide release (Fig. 4, 5). The maximal inhibitory effects of PACAP-38 on capsaicin-induced substance P, CGRP and somatostatin release amounted to 75.4%, 73.3% and 90.0%. In case of EFS-evoked release the maximal inhibition was 80.0%, 87.7% and 67.7%. In case of capsaicin stimulation the EC_{50} values with 95% confidence intervals for substance P, CGRP and somatostatin were 82.9 nM (45.3-151.0 nM), 60.1 nM (22.9-157.2 nM) and 66.9 nM (13.3–135.8 nM), respectively. When EFS was performed, these corresponding EC_{50} data were 92.1 nM (9.9–185.1 nM), 67.8 nM (30.6–150.3 nM) and 20.9 nM (0.5–88.1 nM) (Fig. 6).

Effect of PACAP-38 on acute neurogenic oedema of the mouse ear

In the control group ear thickness increased from 258±15 μm to 375±24 μm within 3 h in response to topical application of 1% mustard oil. PACAP-38 (10, 100 and 1000 μg/kg) significantly diminished mustard oil-induced ear swelling in a dose-dependent manner. The highest applied dose completely abolished the oedema response (Fig. 7). The AUC value calculated on the basis of the time course of ear swelling was 654.2±36.5 units in the control group. Corresponding data in mice treated with 10, 100 and 1000 μg/kg PACAP-38 were 327.2±23.6, 174.2±12.3 and 9.5±3.6, respectively, all significantly smaller than the control (Fig. 7).

Qualitative histological evaluation of the ears showed that 1% mustard oil smearing induced marked oedema formation after 3 hours without significant accumulation of leukocytes. Dose-dependent inhibitory effect of i.p. PACAP-38 on the development of the oedema could also be observed in these histological slides supporting the results of the *in vivo* ear thickness measurement.

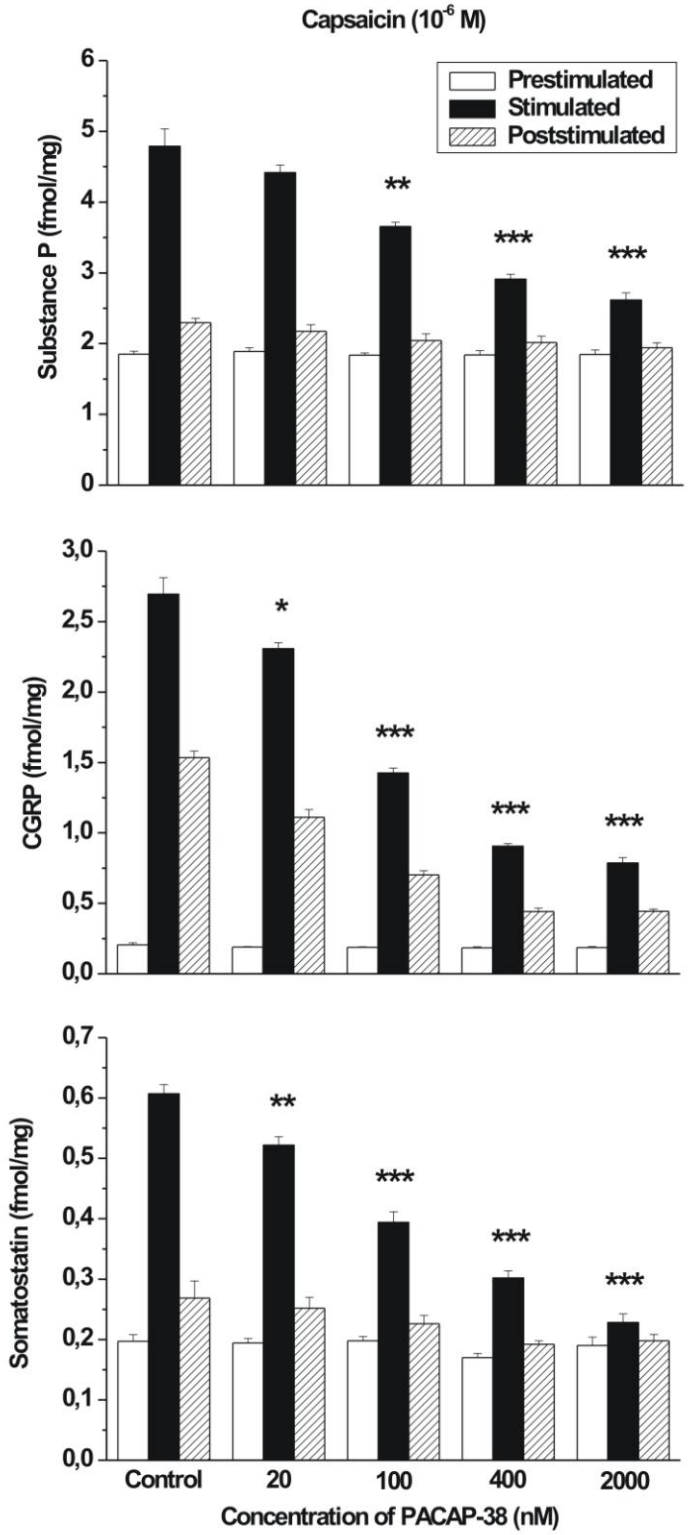

Figure 4. Effect of PACAP-38 on capsaicin-induced release of substance P, CGRP and somatostatin from isolated rat tracheae. Each column represents the mean±SEM concentration of the respective peptide measured in the incubation medium of the pre-stimulated, stimulated and post-stimulated fractions of n=6 experiments (6x2 tracheae). *P<0.05, **P<0.01, ***P<0.001 vs. control stimulated values (Student's t-test for unpaired comparison).

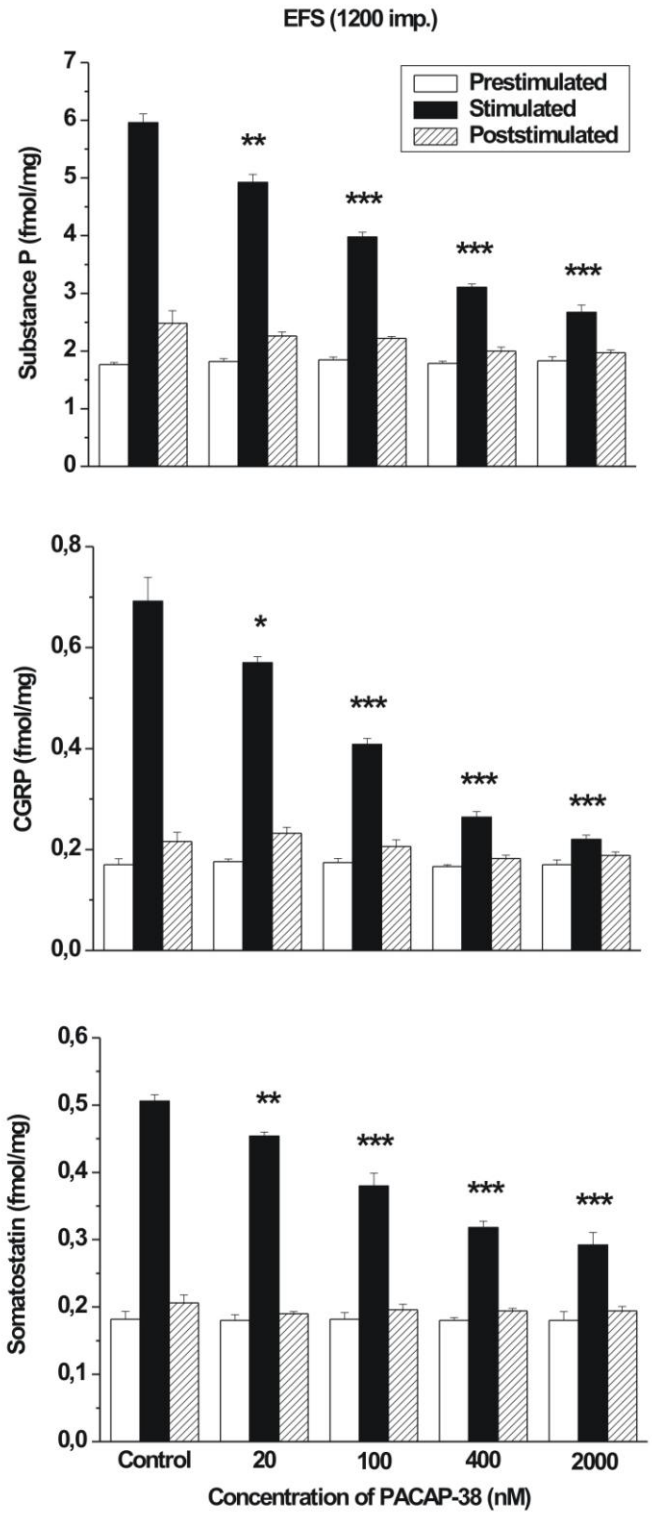

Figure 5. Effect of PACAP-38 on electrical field stimulation (EFS; 40 V, 0.1 ms, 10 Hz, 2 min)-induced release of substance P, CGRP and somatostatin from isolated rat tracheae. Each column represents the mean±SEM concentration of the respective peptide measured in the incubation medium of the pre-stimulated, stimulated and post-stimulated fractions of n=6 experiments (6x2 tracheae). *P<0.05, **P<0.01, ***P<0.001 vs. control stimulated values (Student's t-test for unpaired comparison).

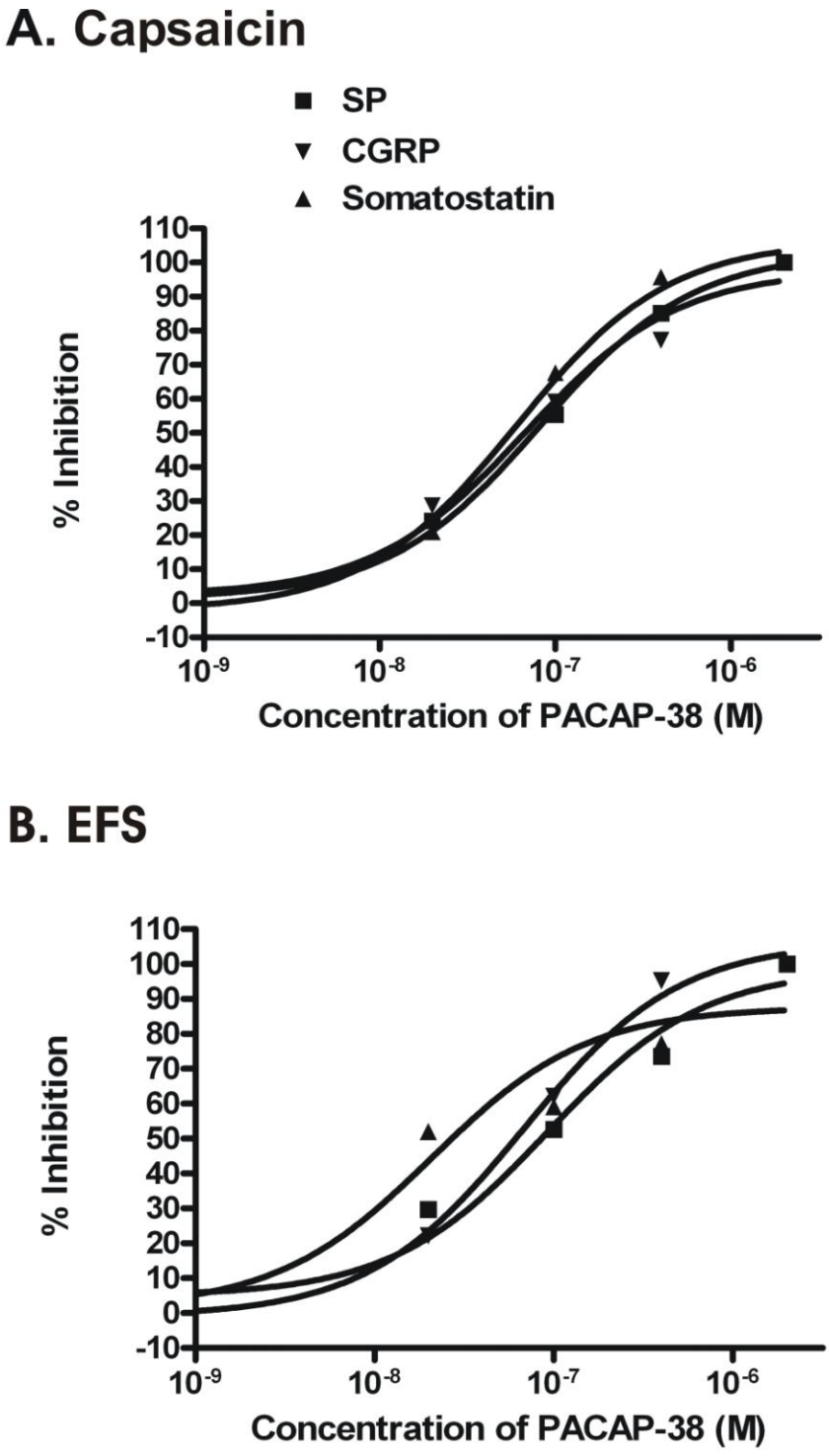

Figure 6. Concentration-response curves demonstrating the inhibitory effect of PACAP-38 on (**a**) capsaicin- and (**b**) electrical field stimulation (EFS)-evoked release of substance P, CGRP and somatostatin from isolated rat tracheae. Concentration of the peptide in the basal, pre-stimulated fraction was subtracted from both the respective stimulated and post-stimulated fractions and then these values were added providing a total stimulation-evoked release. Inhibition evoked by the highest, 2000 nM concentration was considered to be 100% and data points representing mean±SEM relative inhibitory effects (%) were calculated by comparing the results of the studies with PACAP-38 to the control experiments, n = 6.

Effect of PACAP-38 on acute neurogenic inflammation in the skin of the rat hindpaw

Mustard oil-induced Evans blue dye accumulation, as an indicator of neurogenic plasma protein extravasation, in the skin of the acutely denervated rat hindpaw was 302.7±20.5 μg/g wet tissue in the control group. PACAP-38 (100 μg/kg, i.p.) exerted significant, 37.8% inhibition of this neurogenic inflammatory response: Evans blue content of the skin was 200.2±20.4 μg/g.

PACAP-38 inhibits capsaicin- and resiniferatoxin-induced acute neurogenic inflammation in the plantar skin of the rat hindpaw

In the control group Evans blue dye accumulation in the plantar skin of the acutely denervated rat hindpaw was 559.1±74.6 μg/g and 590.3 μg/g wet tissue in response to 100 μl intraplantar RTX (0.1 μg/ml) and capsaicin (100 μg/ml), respectively. Pre-treatment with PACAP-38 (10 μg/kg i.p.) 10 min before the induction of the inflammation exerted 45.7% and 46.4% inhibition on these neurogenic inflammatory responses (Fig. 8).

Effect of PACAP-38 on carrageenan-evoked acute paw oedema in the rat

Intraplantar injection of 100 μl 3% carrageenan induced 43.2%, 50.1% and 57.6% paw swelling in saline-treated rats at 60, 120 and 180 min, respectively. Carrageenan-induced oedema formation was significantly inhibited by 10 μg/kg PACAP-38 i.p. at each time point (Fig. 9). Area under the time-dependent percentage paw swelling curves (AUC) was 65.6±5.1 units in the saline-treated group and 39.9±3.2 units in rats treated with PACAP-38.

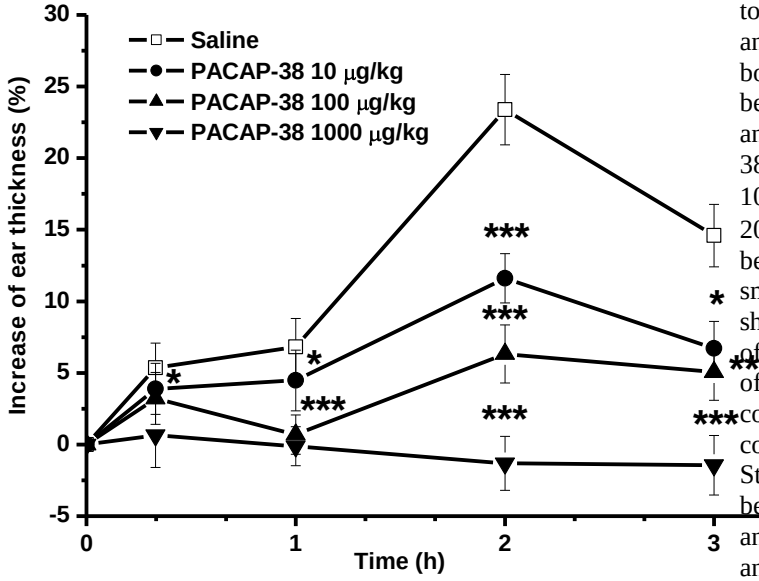

Figure 7. Effect of PACAP-38 on mustard oil-induced ear swelling of the mouse. Mustard oil (1% dissolved in paraffin oil) was applied topically to the outer and inner surfaces of both ears at the beginning of the study and 1 h later. PACAP-38 was injected i.p. (10, 100 and 1000 μg/kg in 200 μl volume) 15 min before both mustard oil smearing. Data are shown as means±SEM of percentage increase of ear thickness compared to the initial control diameters. Statistical comparisons between PACAP-38- and saline-treated animals were made by ANOVA followed by Dunnett's post test, n=12 per group, *P<0.05, **P<0.01, ***P<0.001 vs. saline-treated control.

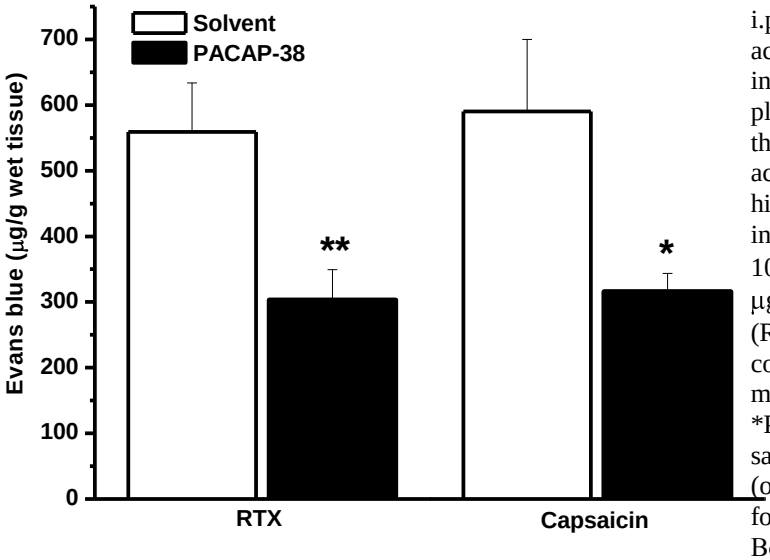

Figure 8. Effect of PACAP-38 (10 μg/kg i.p.) on Evans blue accumulation as an indicator of neurogenic plasma extravasation in the plantar skin of the acutely denervated rat hindpaw induced by intraplantar injection of 100 μl capsaicin (100 μg/ml) or resiniferatoxin (RTX; 0.1 μg/ml). Each column represents the mean±SEM of n=6 rats. *P<0.05, **P<0.01 vs. saline-treated control (one-way ANOVA followed by Bonferroni's test).

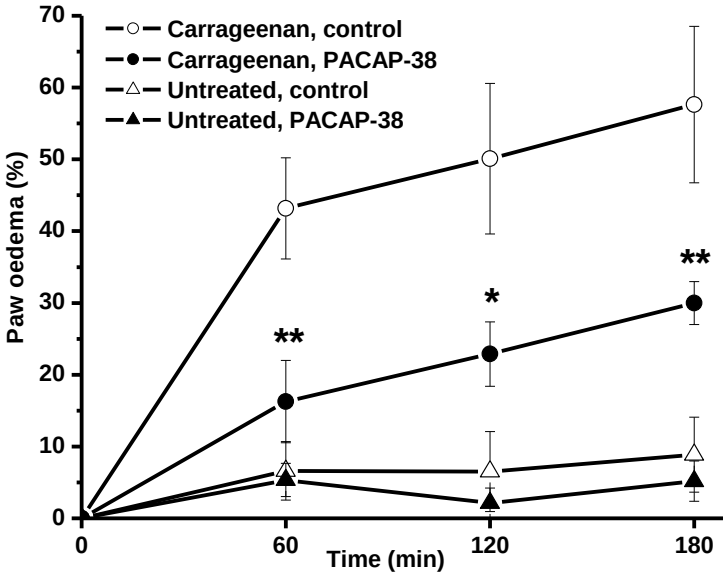

Figure 9. Effect of PACAP-38 (10 μg/kg i.p.) on carrageenan (100 μl 3%)-evoked paw oedema of the rat 60, 120 and 180 minutes after the induction of inflammation. In the control group of rats the same volume of saline was given. Symbols indicate % increase of the volume of the hindpaws as compared to the initial values measured with plethysmometry before carrageenan injection. Each data point represents the mean±SEM of n=6 experiments. *P<0.05, **P<0.01 vs. control (one-way ANOVA followed by Bonferroni's modified t-test).

Discussion

Although immunolocalization of PACAP-38 has been described in capsaicin-sensitive neurons of various species (Moller et al., 1993, Mulder et al., 1994), data about its release and function are ambiguous. PACAP-38 is released by electrical nerve stimulation and capsaicin in the porcine antrum (Tornoe et al., 2001) and in the rabbit eye (Wang et al., 1995; Wang et al., 1997). *In vivo* capsaicin-evoked PACAP release has been shown in the rat spinal cord (Zhang et al., 1997). Our study provides the first evidence that PACAP-38 is released from the peripheral terminals of capsaicin-sensitive afferents in the rat tracheae in response to both capsaicin and electrical field stimulation.

We also demonstrated that PACAP-LI can be detected in the systemic circulation of the rat and *in vivo* stimulation of capsaicin-sensitive nerves by i.v. injection of the ultra-potent TRPV1 receptor agonist RTX induces a two-fold elevation of basal plasma PACAP-38 concentration. Local electrical excitation of capsaicin-sensitive afferents innervating the paw skin was not sufficient to significantly increase PACAP-LI in the blood.

We found that PACAP-38 is able to inhibit both chemically- and electrically-induced substance P, CGRP and somatostatin release from capsaicin-sensitive fibres in a concentration-dependent manner. The maximal inhibitory action of PACAP-38 on sensory neuropeptide release was 70-90% which suggests higher potency than that of similar concentrations of nociceptin and the heptapeptide somatostatin analogue TT-232 (45-65% inhibition). The efficacy of PACAP is similar to the efficacy of nociceptin or somatostatin (Helyes et al., 1997; Helyes et al., 2001). It has previously been described that PACAP-38 suppressed vagal nerve stimulation-induced non-adrenergic non-cholinergic tracheal contractions without affecting the effect of exogenously administered SP. The authors concluded that PACAP may inhibit SP release from nerve terminals (Shigyo et al., 1998), which is supported by our results. Interestingly, in the isolated, perfused porcine antrum PACAP-38 lead to somatostatin and SP release, the source of which has not been elucidated (Tornoe et al., 2001). These data might reveal species- and organ-dependent differences in the actions of PACAP.

Our data demonstrate that systemic administration PACAP-38 inhibits neurogenic inflammation in the mouse *in vivo* in a dose-dependent manner. Furthermore, i.p. PACAP-38 injection significantly diminished mustard oil-evoked plasma protein extravasation in the skin of the rat hindpaw. Since mustard oil-induced release of pro-inflammatory neuropeptides is exclusively responsible for ear swelling in this pure neurogenic inflammatory model, the inhibition of their outflow by PACAP-38 results in the decrease of oedema formation. Ear sections representing the histopathological changes 3 h after mustard oil smearing revealed that marked oedema developed in the connective tissue without significant number of accumulated leukocytes. These findings are supported by our earlier data demonstrating that mustard oil-evoked neutrophil accumulation appears 6 h after challenge. However, capsaicin-sensitive sensory fibres are not involved in the cellular phase of the inflammatory reaction (Bánvölgyi et al., 2004).

We also provided evidence for the ability of i.p. injected PACAP-38 to inhibit capsaicin- or RTX-induced acute neurogenic plasma protein extravasation in the plantar skin of the rat hindpaw. Both ligands selectively activate TRPV1 receptors on capsaicin-sensitive nerves, but RTX has a thousand-fold greater potency (Szállási & Blumberg, 1989; Lazar et al., 2006). Our results also showed that thousand times smaller concentration of RTX induced similar Evans blue leakage to capsaicin.

PACAP also decreased carrageenan-induced inflammation, in which both neurogenic and non-neurogenic components are involved (Romero et al., 2005). Several cellular and neural mediators, such as bradykinin and tachykinins, are implicated in the exsudative response evoked by carrageenan, but complement factors and histamine seem to have minor roles (Dawson et al., 1991; Romero et al., 2005). Concerning the involvement of capsaicin-sensitive fibres is not completely consistent. Sensory neurogenic components have been shown to play a pivotal role in carrageenan-evoked joint (Lam & Ferrell, 1989) and pleural inflammation (Raychaudhuri et al., 1991), as well as in cutaneous neutrophil accumulation (Cao et al., 2000). Others found that capsaicin-sensitive sensory fibres are not important in carrageenan-induced plasma extravasation and oedema in the skin (Zhou et al., 1998), but the integrity of sympathetic fibres is required (Coderre et al., 1989).

The anti-inflammatory effect of PACAP-38 in these different models is likely to be explained by its ability to diminish the release of SP and CGRP from sensory nerves, as well as other inflammatory mediators from sympathetic fibres or cellular sources.

Besides these pre-junctional actions, its direct inhibitory effect on plasma extravasation and oedema formation at the level of the vascular endothelium can also be assumed. This possibility is supported by the presence of PAC1 receptors on endothelial cells (Abu-Hamdan et al., 2006) and the direct anti-apoptotic effects observed in these cell cultures after PACAP administration (Rácz et al., 2007). Numerous data indicate the localization of PACAP and its receptors on blood vessels and its relaxing action on vascular smooth muscle (Fahrenkrug et al., 2000; Dalsgaard et al., 2003), but this vasodilatory effect has been shown to be independent of the endothelium (Warren et al., 1991).

PACAP-38 functions as an immunomodulator, and the majority of the studies report on its anti-inflammatory actions (Ganea & Delgado, 2002; Abad et al., 2006). Intraperitoneal administration of PACAP-38 in mice has been shown to reduce inflammatory changes in the collagen-induced arthritis model by modulating several inflammatory soluble factors (Abad et al., 2001) and to ameliorate the clinical manifestations of experimental autoimmune encephalomyelitis by suppressing the function of antigen presenting cells (Kato et al., 2004). Clinical studies have also suggested that PACAP is involved in inflammatory processes in psoriasis (Steinhoff et al., 1999). However, there are also data on its pro-inflammatory actions, like vasodilation and oedema formation in the rabbit eye (Wang et al., 1995; Wang et al., 1997). Furthermore, intradermal injections of low PACAP-38 concentrations evoked plasma extravasation in the rat and rabbit skin by inducing histamine release from activated mast cells and directly dilating blood vessels (Warren et al., 1993; Cardell et al., 1997). Based on these data it seems that the effects of PACAP-38 depends on its route of administration (central versus peripheral) as well as the pathological mechanisms of the inflammatory models used.

In summary, 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

Introduction

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 (Young et al., 1983; Carlson et al., 1985; De Young et al., 1989; Kuchera et al., 1993; Garrido et al., 2004; Huang et al., 2006). In these reports ear swelling, neutrophil infiltration, histopathological changes and local cytokine concentration were the indicators of inflammatory processes. PMA, when administered topically to the mouse ear, induces a pronounced inflammatory response mediated by protein kinase C (PKC) (Kuchera et al., 1993). Neurogenic components were also evidenced in PMA-induced inflammation in murine ears by vanilloid-pre-treated animals. PMA induced early oedema response in the mouse ear was strongly inhibited by pre-treatment with RTX (Szállási & Blumberg, 1989).

Phosphorylation and consequent sensitization/activation of TRPV1 receptors by PKC has been established by numerous studies (Cesare et al., 1999; Chuang et al., 2001; Varga et al., 2006).

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

Animals

Experiments were performed on male C57BL/6 TRPV1 receptor gene knockout mice (TRPV1-/-) and their wild-type counterparts (TRPV1+/+). The ancestors of the transgenic mice were donated by Dr. J. B. Davis, Neurology and GI Centre of Excellence for Drug Discovery, GlaxoSmithKline, Research and Development Ltd., New Frontiers Science Park, Essex, Harlow, UK (Davis et al., 2000). TRPV1 knockout mice were fully backcrossed into C57BL/6 mice and these animals were used to generate TRPV1 knockout colonies. TRPV1-/- animals showed normal growth and behavioural characteristics. Animals were bred in the Animal House of Pécs University under pathogen free condition at 24-25 ºC in climatically controlled environment, given standard diet and water *ad libitum*.

PMA and acetone treatment

TRPV1 receptor knockout and wild-type mice were anaesthetized by ketamine (100 mg/kg i.p., repeated if necessary) and xylazine (5 mg/kg i.p.). Right ears of the animals were smeared with PMA dissolved in acetone. PMA (1.25 μg) was applied in 10-10 μl volume to the inner and outer surfaces of the right ear. Left ears were smeared with 10-10 μl acetone. Separate groups of animals received acetone on both ears or PMA on the right ear and the left ear remained untreated. At the end of the incubation period animals were killed by cervical dislocation, ears were dissected for histology and myeloperoxidase assay.

Systemic resiniferatoxin pre-treatment

Neuropeptides from capsaicin-sensitive primary afferents were depleted by systemic resiniferatoxin 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. Mice received aminophylline (1.92 mg/kg), terbutaline (20 μg/kg) and atropine (20 μg/kg) i.p. just before RTX treatment to avoid respiratory complications. Experiments were carried out 14 days after RTX pre-treatment. The effect of pre-treatment was checked by the eye wiping test. Desensitization was declared successful, if 0.1% capsaicin dropped into the eye did not cause wiping movements.

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). In a group PMA was applied on the right, capsaicin-treated ear, and the left ear received acetone. In another animal group acetone treatment was performed on the left, capsaicin-treated side and PMA was used on the right, ethanol treated ear.

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 6^{th} and 12^{th} 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. Normal goat IgG was used as control. 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 (left ears or both ears in a separate group of animals) or PMA (right ears) and after the treatments in different time points as described previously. We used an engineers' micrometer (0.01 mm accuracy). Data were expressed as % increase of ear thickness compared to the initial pre-challenge values.

Myeloperoxidase assay

Myeloperoxidase activity correlates to the number of accumulated neutrophils. Samples were collected 12 hours after acetone and PMA challenge and stored at -20 ºC. Ears were weighed, chopped into small pieces and homogenised in phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide. Two ml buffer was used for each ear. The homogenate was centrifuged at 10000 g, 4 ºC 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'-tetramethyl-benzidine at room temperature. The optical density (OD) at 620 nm was measured at 5 min intervals using a microplate reader. The reaction rate was calculated and a calibration curve was produced with the reaction rate plotted against MPO content using standard samples (MPO from human leukocytes).

Histology

Samples were fixed in 4% buffered paraformaldehyde and embedded into paraffin. 5 μm cross sections were made and stained with haematoxylin and eosin. Digital photographs were taken of the slides by an Olympus BX51 microscope and Olympus DP50 camera. To reflect the severity of inflammation, the extent of the oedema, formation of microabscesses after necrosis of hair follicles and sebaceous glands and

number of accumulated mononuclear and polymorphonuclear cells were scored. Composite scores for the different experimental groups were calculated.

Measurement of IL-1β concentration

*S*amples 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 ºC for 10 minutes and 500 μl of supernatants were collected. IL-1β content was determined by BD OptEIA ELISA set.

Statistics

Results are expressed as mean ± standard error of mean (SEM). Comparisons between different groups of animals were performed by ANOVA followed by Bonferroni's test. Probability values $p<0.05$ were accepted as significant.

Ethics

Ethical guidelines declared in the previous chapter were observed when executing the present studies.

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. This decreased to approximately 46-62% at 24 hours. In the first 4 hours these acetone treated ears did not show detectable swelling. Initial ear thickness values of TRPV1+/+ and -/- animals were 272.96±2.66 μm and 266.52±4.89 μm respectively for the acetone treated left ears (Fig. 10). PMA induced 163-196% ear swelling both in TRPV1+/+ and TRPV1-/- mice during the 24 hour measurement period compared to the pre-stimulated baseline thickness. Pre-stimulated ear thickness values of the PMA-treated right ears were 276.67±2.72 μm for TRPV1+/+ and 270.00±4.12 μm for TRPV1-/- mice (Fig. 10).

Systemic pre-treatment of TRPV1+/+ animals with the ultra-potent capsaicin analogue resiniferatoxin ameliorated contralateral acetone-induced ear swelling at every time point (0-24 h) compared to TRPV1+/+ mice. Oedema was reduced by 33-83%. Baseline ear thickness values of the left, acetone-treated ears were 254.62±2.43 μm (Fig. 11). PMA-induced ear oedema formation was inhibited by 30-50% particularly in the early phase (0-6 h) of inflammation. Initial values of the right, PMA-treated ears were 247.77±3.95 μm (Fig. 11).

Only 12-20% increase in ear oedema was observed in a separate group of mice wherein both ears were treated with acetone (Fig. 10). Interestingly, 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 (data are not shown).

Neither genetic lack of TRPV1 receptors nor systemic RTX pre-treatment produced significant changes in bilateral acetone treatment-induced ear oedema. Baseline values were 317.78±5.21 μm, 375.42±1.20 μm and 297.50±5.041 μm for TRPV1+/+, -/- and TRPV1+/+ RTX-pre-treated mice, respectively (Fig. 11).

In a separate animal group local capsaicin-desensitization was performed on the acetone treated left side, but not on the PMA treated right ear of TRPV1+/+ mice. In spite of strong inflammatory changes at the PMA-treated side, acetone evoked only moderate ear swelling. Initial value of the pre-treated left ears was 303.33±4.94 μm and of the PMA-treated right ears was 303.33±1.20 μm. Baseline values of the control acetone (left) and PMA-treated (right) ears were 295.00±6.45 μm and 320.00±16.83 μm, respectively (Fig. 12a). 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 approximately 74-91% ear swelling. Baseline values of the acetone treated and the capsaicin-PMA treated ears were 303.33±4.94 μm and 290.00±4.47 μm. Initial values of control acetone (left) and PMA-treated (right) ears were 286.67±11.16 μm and 278.33±4.77 μm (Fig. 12b).

Treatment of TRPV1+/+ mice with ibuprofen (70 mg/kg), a cyclooxygenase inhibitor, completely prevented ear swelling induced by contralateral acetone application on the left (Fig. 13). Ibuprofen treatment did not cause significant change in PMA-oedema on the right. Initial values of the ibuprofen-treated acetone- and PMA-smeared ears were 320.00±13.17 μm and 321.67±13.27 μm. Baseline control acetone (left) and PMA-treated (right) values were 300.00±5.77 μm and 290.00±11.55 μm.

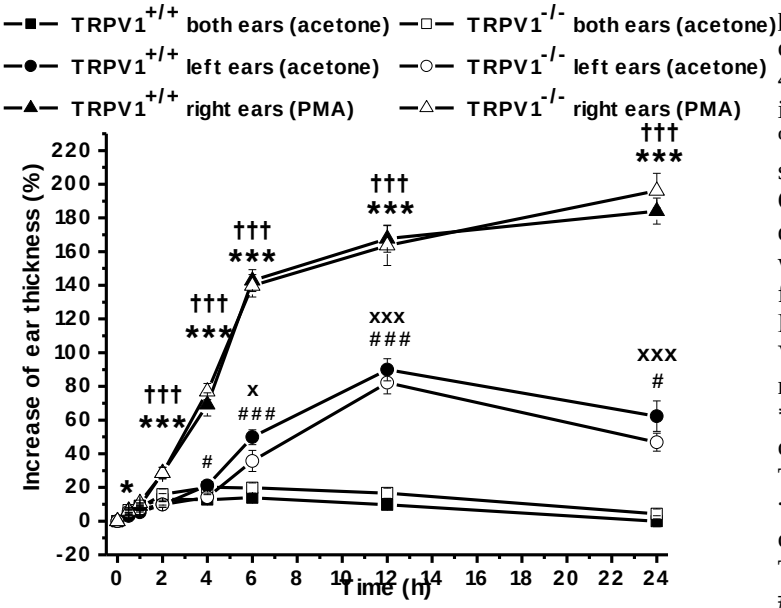

Figure 10. Contralateral acetone and bilateral acetone treatment in TRPV1+/+ and -/- mice. Measurements were performed before challenge and 0.5, 1, 2, 4, 6, 12, 24 hours after it. Data are expressed as % change from pre-stimulated values. Comparisons between different treatments were made by ANOVA followed by Bonferroni's test. Values are means±SEM, n=6-27. *P<0.05, ***P<0.001 vs. contralateral acetone in TRPV1+/+ animals. †††P<0.001 vs. contralateral acetone in TRPV1-/- animals. #P<0.05, ###P<0.001 vs. bilateral acetone in TRPV1+/+ animals. xP<0.05, xxxP<0.001 vs. bilateral acetone in TRPV1-/- animals.

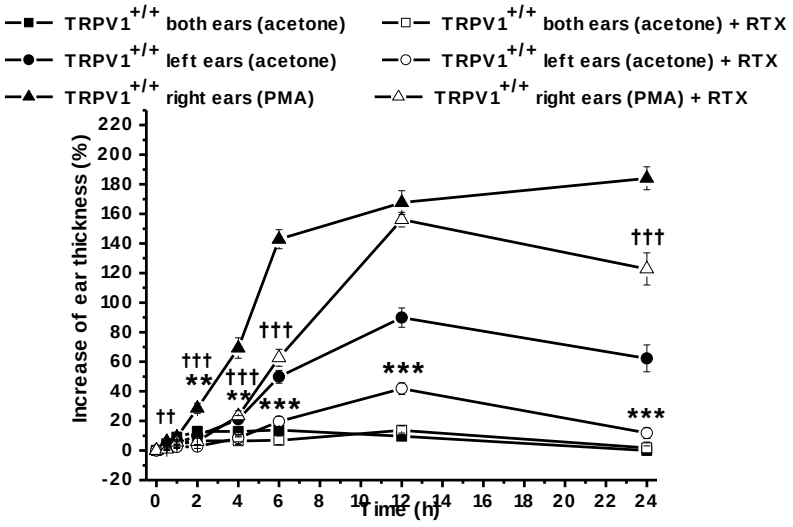

Figure 11. PMA, contralateral acetone and bilateral acetone treatment induced ear swelling in TRPV1+/+ RTX-pre-treated and control animals. Measurements were performed before challenge and 0.5, 1, 2, 4, 6, 12, 24 hours after it. Data are expressed as % change from pre-stimulated values. Comparisons between different groups were made by ANOVA followed by Bonferroni's test. Values are means±SEM, n=6-27. **P<0.01, ***P<0.001 vs. contralateral acetone in TRPV1+/+ animals. ††P<0.01, †††P<0.001 vs. PMA in TRPV1+/+ animals.

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 compared to naïve controls. Contralateral acetone treatment did not cause significant neutrophil accumulation in TRPV1+/+ and RTX-pre-treated mice (Fig. 14).
Strongly significant increase in MPO activity was observed in PMA-challenged right ears after 12 hours indicating a marked increase in the accumulation of polymorphonuclear leukocytes and other myeloperoxidase positive cells. Neither systemic RTX pre-treatment, nor deletion of TRPV1 receptors caused significant inhibition in MPO activity compared to their controls (Fig. 14).

Histological findings

The diameter of the ear sections determined by analysis of the histological slides correlated with the ear thickness data. The highest number of microabscesses due to inflammation/necrosis of hair follicles and sebaceous glands 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, but not in wild-type ones. 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. Representative light micrographs demonstrate most prominent histopathological changes (Fig. 15).

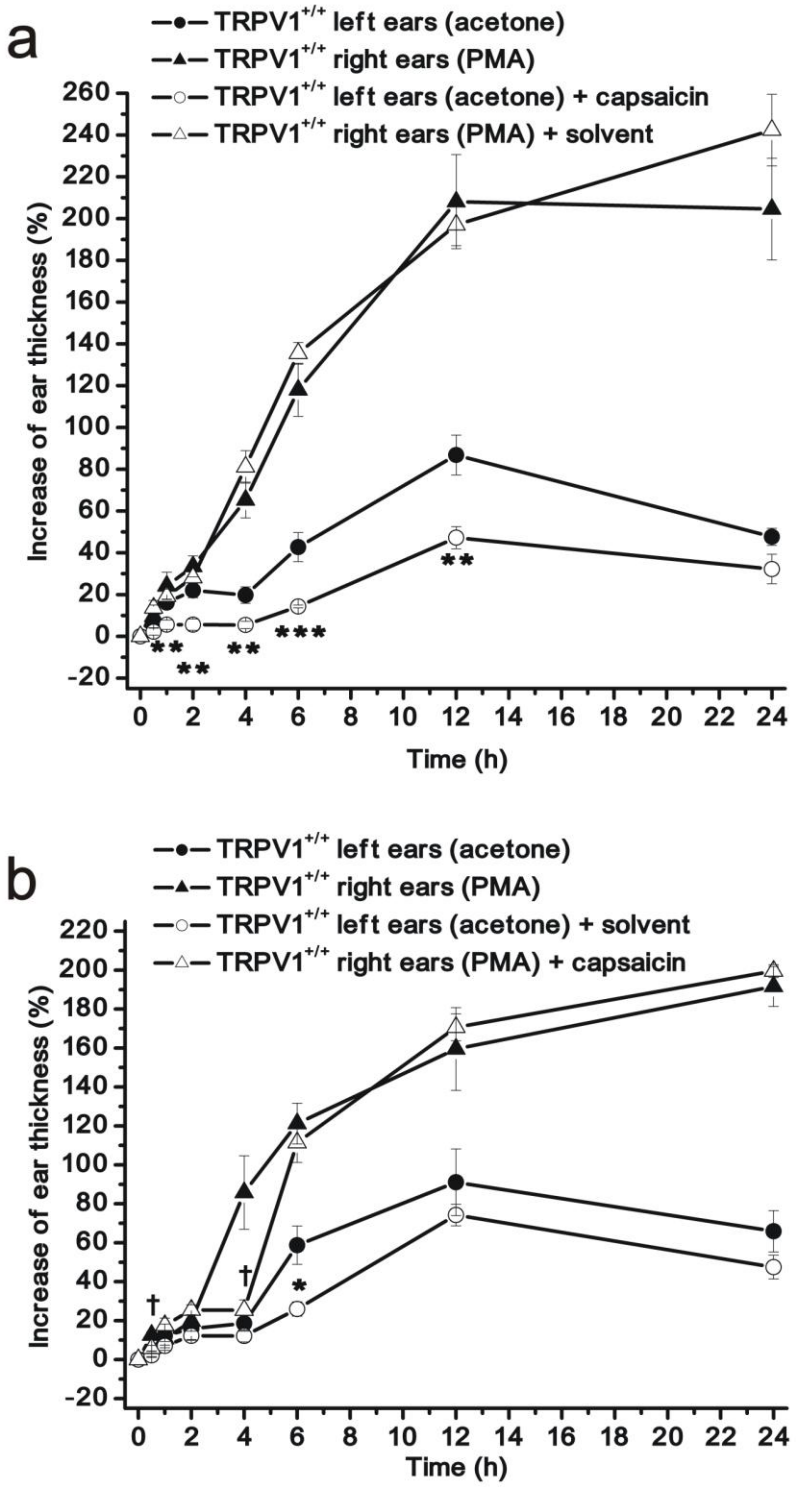

Figure 12. Effect of local capsaicin desensitization on PMA (right) and contralateral acetone (left) induced ear oedema. (**a**) Capsaicin desensitization of the left, acetone-treated ear. (**b**) Capsaicin desensitization of the right, PMA-treated ear. Ear thickness measurements were performed before challenge and 0.5, 1, 2, 4, 6, 12, 24 hours after challenge. Data are expressed as % change from pre-stimulated values. Comparisons between different treatments were made by ANOVA followed by Bonferroni's test. Values are means±SEM, n=3-6. *P<0.05, **P<0.01 vs. TRPV1+/+ control animals that received contralateral acetone (left side) treatment. †P<0.05 vs. TRPV1+/+ control animals treated with PMA (right side).

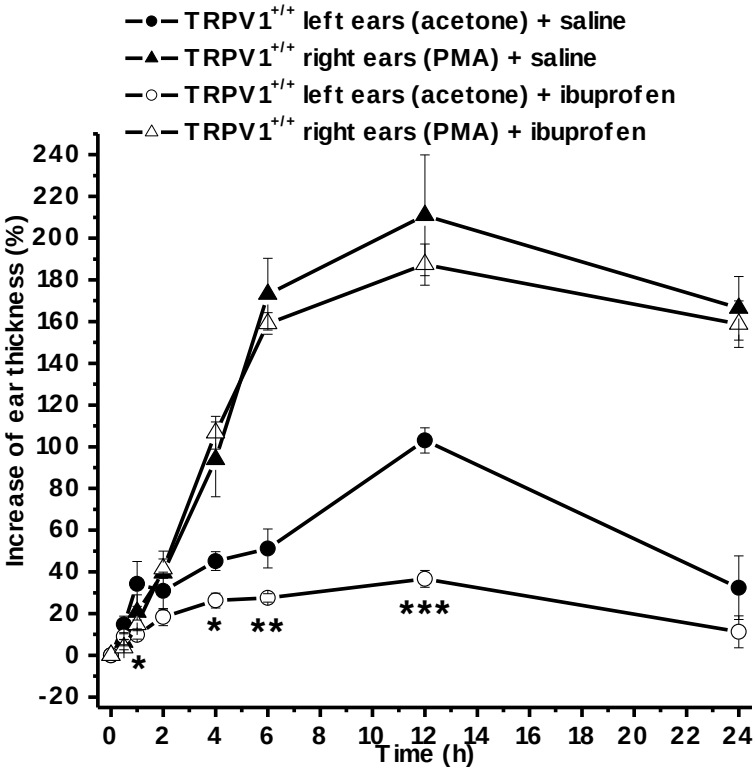

Figure 13. PMA (right) and contralateral acetone (left) induced ear swelling in TRPV1+/+ ibuprofen treated and control animals. Ear thickness measurements were performed before challenge and 0.5, 1, 2, 4, 6, 12, 24 hours after challenge. Data are expressed as % change from pre-stimulated values. Comparisons between different groups were made by ANOVA followed by Bonferroni's test. Values are means±SEM, n=3-6. *P<0.05, **P<0.01, ***P<0.001 vs. contralateral acetone (left) and saline-treated TRPV1+/+ animals.

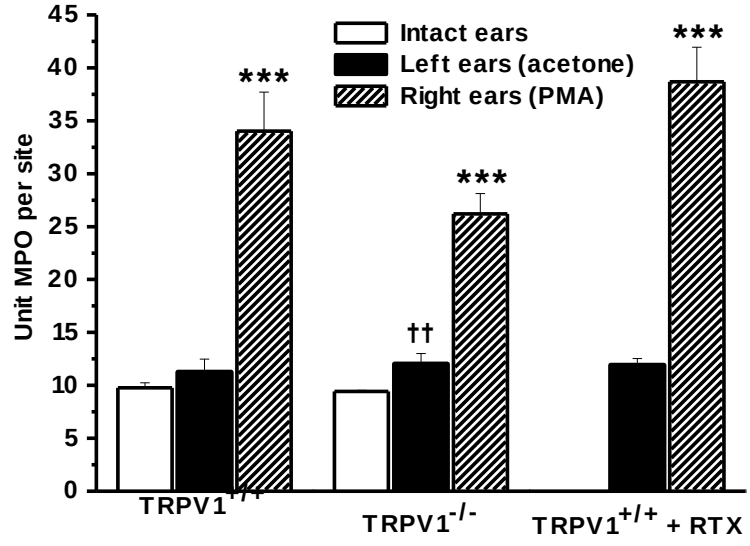

Figure 14. PMA (right ears) and contralateral acetone (left ears) treatment induced neutrophil accumulation in TRPV1+/+, TRPV1-/- and TRPV1+/+ RTX-pre-treated animals. Measurements were performed 12 hours after challenge. Data are expressed as MPO content per ear. Statistical evaluation was performed by ANOVA followed by Bonferroni's test. Values are means±SEM, n=6-8. ***P<0.001 vs. contralateral acetone (left). ††P<0.01 vs. intact.

Effect of PMA and acetone treatment on local IL-1β content of the mouse ear

Increased IL-1β level was measured on the acetone treated left side in PMA-treated (right ears) animals. Bilateral acetone treatment did not cause IL-1β elevation compared to the naïve controls, either in the ears of TRPV1+/+ or TRPV1-/- animals (Fig. 16). Furthermore, PMA treatment on the right side resulted in similarly elevated local IL-1β levels in the ears of TRPV1+/+ and TRPV1-/- mice (Fig. 16).

Effect of systemic anti-IL-1β antibody treatment on PMA- and acetone-induced ear oedema

Pre-treatment with anti-IL-1β antibody did not ameliorate the potentiating effect of PMA on contralateral (left side) acetone-induced ear swelling at any time points compared to the goat IgG-treated control. The antibody treatment also did not diminish PMA-induced right ear inflammation.

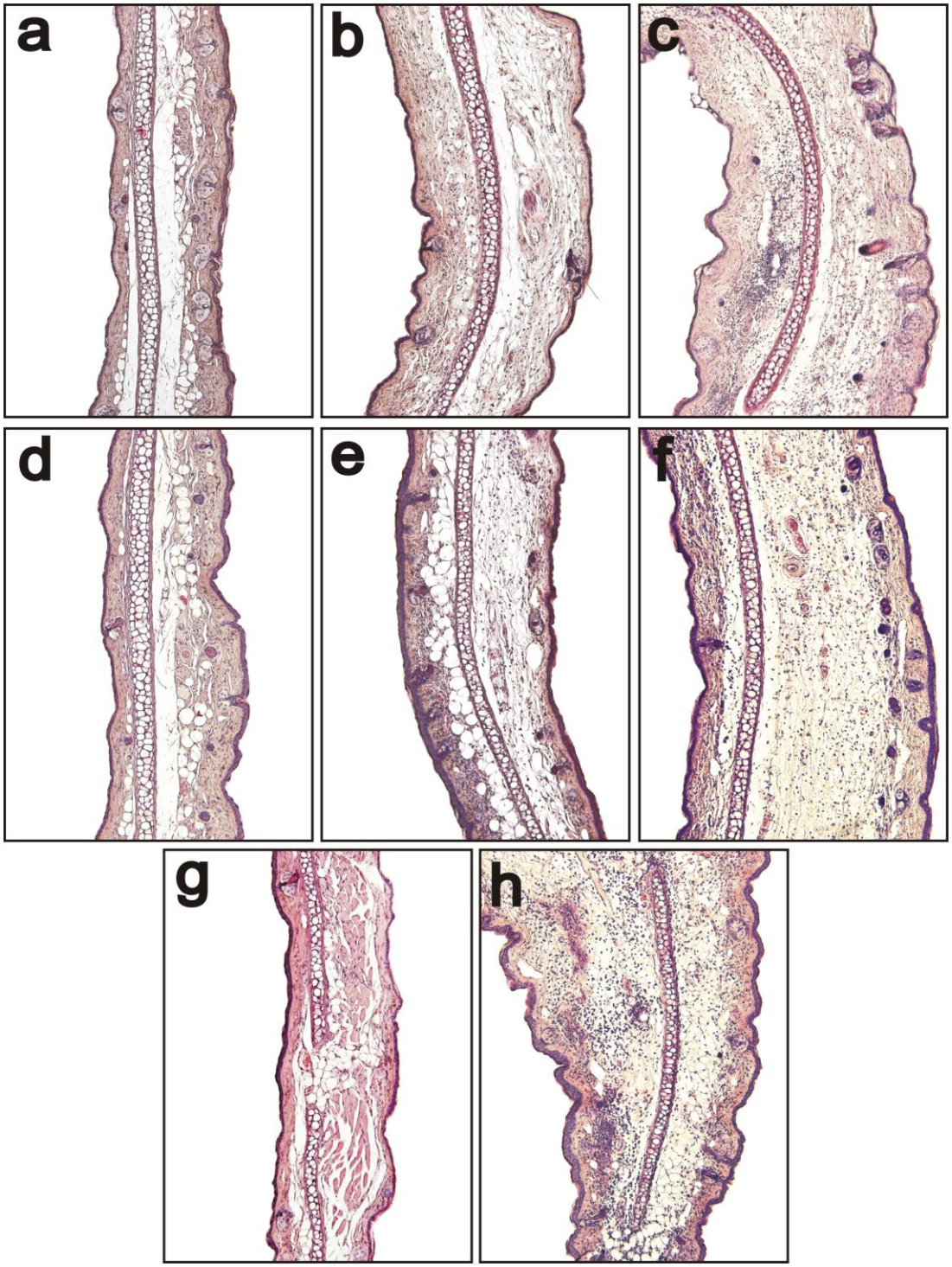

Figure 15. Representative light micrographs (100 X). (**a**) Bilateral acetone, (**b**) contralateral acetone (left ear) and (**c**) PMA (right ear) treatment in TRPV1$^{+/+}$ animal. (**d**) Bilateral acetone, (**e**) contralateral acetone (left ear) and (**f**) PMA (right ear) treatment in TRPV1$^{-/-}$ subject. (**g**) Contralateral acetone (left ear) and (**h**) PMA (right ear) application in TRPV1$^{+/+}$ RTX-pre-treated animal.

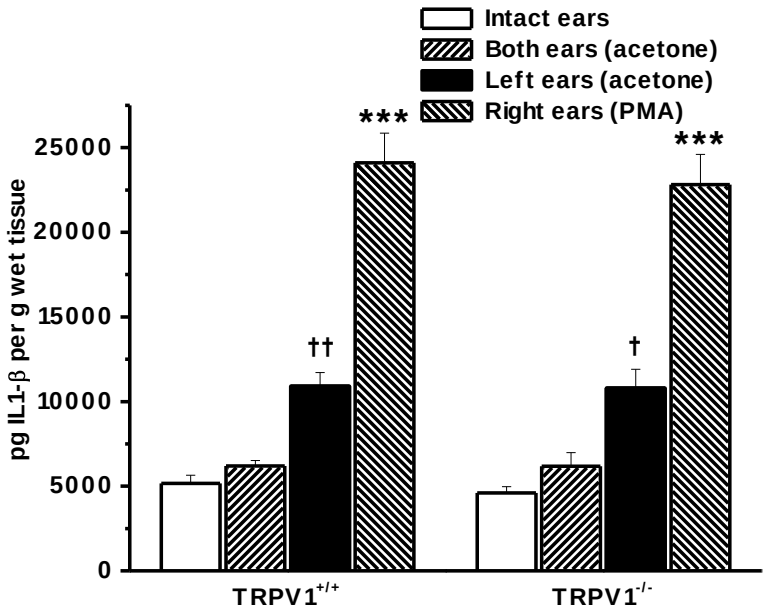

Figure 16. IL-1β content of PMA (right), contralateral acetone (left) and bilateral acetone-treated mouse ears. IL-1β detection was performed 12 hours after challenge. Data are expressed as pg IL-1β per grams of ear samples. Comparisons between different groups were made by ANOVA followed by Bonferroni's test. Values are means±SEM, n=6-19. ***P<0.001 vs. contralateral acetone (left) treatment. †P<0.05, ††P<0.01 vs. bilateral acetone application.

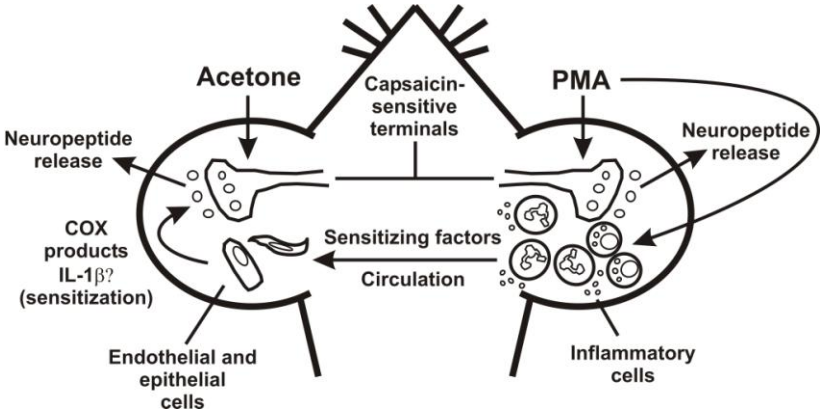

Figure 17. Proposed mechanism of sensitization exerted by PMA on the capsaicin-sensitive nerve terminals of the contralateral ear. PMA stimulates capsaicin-sensitive nerve terminals resulting in neuropeptide release and neurogenic oedema in the right ear. It also induces the accumulation of inflammatory cells secreting different inflammatory mediators (kinins, cytokines) which enter the circulation and reach the contralateral left ear wherein they trigger prostanoid synthesis. Prostanoids may sensitize capsaicin-sensitive fibres to acetone. Concurrent acetone application to the site of sensitization leads to neurogenic oedema.

Discussion

Acetone is widely used as an inert solvent of PMA (Young et al., 1983; De Young et al., 1989; Szállási & Blumberg, 1989; Bermejo et al., 1998; Griswold et al., 1998; Garrido et al., 2004), but in our study contralaterally applied acetone revealed inflammatory potential after 4 hours. It increased ear thickness by 85%, but only when the ipsilateral ear was treated with PMA. Acetone treatment had only slight effect without application of PMA to the other ear. Furthermore, the intact ear contralateral to PMA showed significantly less swelling indicating that sensitization by distant PMA application is needed to provoke marked acetone-induced ear oedema (Fig. 17). Since oedema formation was not altered in TRPV1 gene-deleted mice, but significantly reduced by RTX pre-treatment and local capsaicin desensitization of the acetone-treated ear, we suppose that acetone acts on the capsaicin-sensitive nerve endings a TRPV1-independent manner. Capsaicin pre-treatment of the PMA-treated ear did not ameliorate contra lateral acetone-induced ear swelling and inhibited PMA-evoked ear inflammation only at an early time point. These data together with the finding that contralateral acetone treatment causes oedema after 3-4 hours suggest that the sensitizing effect of PMA is mediated by substances from inflammatory cells accumulating at the PMA-treated side and not by capsaicin-sensitive afferents of this ear. It is possible that mediators released from the accumulated inflammatory cells reach the other ear via the circulation and sensitize capsaicin-sensitive nerve terminals.

The fact that PMA-induced ear inflammation develops in TRPV1 knockout animals similarly to wild type mice underlines that phosphorylation and sensitization of TRPV1 receptor by PKC is not pivotal in this case. On the other hand, according to the present and earlier studies (Szállási & Blumberg, 1989) systemic pre-treatment with RTX inhibited PMA ear oedema during the first 4 hour period. Local desensitization with capsaicin also inhibited this early phase of PMA-induced ear inflammation. Since the late PMA-oedema and cellular infiltration were not affected by RTX or capsaicin pre-treatment, neurogenic mediation cannot be supposed.

Together with similar findings on nociception (Ferreira et al., 1988; Follenfant et al., 1989), previous data indicate that interleukin-1 beta sensitizes afferent nerve fibres to both chemical and mechanical noxious stimuli (Herbert et al., 1995). Although local

IL-1β concentration was increased in the acetone-treated ear, anti-IL-1β antibody did not attenuate contralateral acetone-evoked ear swelling. These data provide evidence that IL-1β from the PMA-treated ear and the locally released cytokine on the acetone-treated side do not play pivotal role in the potentiating effect on nerve endings. Since the administration of the antibody also did not influence PMA-induced ear swelling, we conclude that IL-1β is not essential in this inflammatory model.

The attenuating effect of the cyclooxygenase inhibitor ibuprofen on contralateral acetone-evoked ear swelling confirms that prostanoids play a pivotal role in this phenomenon. Ibuprofen treatment did not diminish PMA-oedema. It was described earlier that this cyclooxygenase inhibitor is more potent against cellular events of PMA-inflammation, than against oedema formation (De Young et al., 1989).

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-1β 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-1β 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

Introduction

TRPV1 expressing afferent nerves in the gastrointestinal tract were described by some authors among extrinsic sensory neurons only in rodents (Patterson et al., 2003; Ward et al., 2003). Others found TRPV1-like immunoreactivity in intrinsic enteric neurons of the guinea pig, pig and humans (Poonyachoti et al., 2002; Anavi-Goffer & Coutts, 2003; Chan et al., 2003). Under physiological conditions TRPV1 expressing afferents play an important role in the regulation of gastrointestinal circulation, secretion, mucosal homeostasis, motility and nociception (Holzer & Barthó, 1996; Holzer & Maggi, 1998). Involvement of capsaicin-sensitive nerve endings and TRPV1 receptor in inflammatory bowel disease was also investigated. 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). Pathogenetic basis of dextran sulphate-induced colitis is progressive crypt loss in the colonic mucosa (Cooper et al., 1993) and alterations of luminal bacterium species. Activation of inflammatory cells may also contribute (Verdu et al., 2000; Rath et al., 2001; Setoyama et al., 2003; Rachmilewitz et al., 2004). TNBS induces colitis by working as a hapten (Cavani et al., 1995; Neurath et al., 1995).

Topical co-administration of 640 μmol/l capsaicin inhibited TNBS-induced inflammation in rats (Goso et al., 1993) showing the anti-ulcerative properties of neuropeptides released by capsaicin from sensory fibres. Orally administered capsaicin was also protective against oral dextran sulphate sodium-induced colitis in rats (Okayama et al., 2004). In contrast with these findings 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 ending by capsaicin pre-treatment exacerbated inflammatory changes in TNBS, DSS and various other models (Leung, 1992; Reinshagen et al., 1994, 1996; Eliakim et al., 1995;

McCafferty et al., 1997; 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. For the experiments TRPV1 receptor gene knockout mice or systemic pre-treatment with a higly potent capsaicin analogue, resiniferatoxin, were used.

Methods

Animals

Experiments were executed on male C57BL/6 TRPV1 receptor gene knockout mice (TRPV1-/-) and their wild-type counterparts (TRPV1+/+). TRPV1-/- animals were purchased from The Jackson Laboratory, USA. TRPV1 knockout mice were fully backcrossed into C57BL/6 mice and these animals were used to generate TRPV1 knockout colonies. TRPV1-/- animals showed normal growth and behavioural characteristics. All animals were bred in the Animal House of Pécs University under pathogen free condition at 24-25 ºC in climatically controlled environment, given standard diet and water *ad libitum*.

Induction of colitis

DSS (2%) dissolved in millipore water was administered orally to mice *ad libitum* for 6 or 5 days. The standard regimen of 5% DSS for 7 days was reduced, because of the experienced excessive animal loss. Control animals received millipore water for 6 days. 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 and put into 4% buffered paraformaldehyde for histological examination. The remaining samples were put on -20 °C for myeloperoxidase assay.

Systemic resiniferatoxin pre-treatment

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

Disease activity index

Weight loss compared to initial body weight, 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. Occult rectal bleeding was detected by a commercial test using modified guaiac-method. Survival analysis of TRPV1+/+, TRPV1-/- and TRPV1+/+ RTX-pre-treated DSS drinking animals was also performed.

Myeloperoxidase assay

Samples were stored at -20 ºC. Colon segments were weighed, chopped into small pieces and homogenised in 1 ml of 50 mM potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (pH 6). The homogenate was centrifuged at 10000 g, 4 ºC for 10 minutes. 400 μl of the supernatant was collected. Myeloperoxidase activity was determined as described above.

Histology

Four mm wide rings from proximal, intermediate and distal colon segments were fixed in 4% buffered paraformaldehyde and embedded into paraffin. 5 μm cross sections were made and stained with haematoxylin and eosin. Digital micrographs were taken by an Olympus BX51 microscope and Olympus DP50 camera. Crypt height, severity and extent of inflammation were scored as described earlier (Mantyh et al., 1996; Tessner et al., 1998; Kihara et al., 2003). Histological evaluation was performed by AnalySIS software (Soft Imaging System).

Statistics

Results are expressed as mean ± standard error of mean (SEM). Statistical evaluation of data was fulfilled with Student's t-test and ANOVA followed by Bonferroni's test. Survival analysis was performed by logrank test. Probability values $p<0.05$ were accepted as significant.

Ethics

Ethical guidelines proclaimed previously were observed when performing these studies.

Results

Disease activity index

By the end of the 6 day regimen severe bloody diarrhoea was developed in each TRPV1+/+ animal. Two percent DSS solution led to death of 57.14% of animals in this strain. Genetic lack of TRPV1 receptor did not affect the disease activity index (Fig. 18a). All TRPV1-/- mice showed also bloody diarrhoea and 38.46% of them died in 6 days. At the RTX-pre-treated TRPV1+/+ animal group the regimen had to be reduced to 5 days, because of enormous loss of animals (91.67%). Surprisingly, nevertheless the striking sudden loss of animals on the 6^{th} day, systemic RTX pre-treatment did not change disease activity index values compared to TRPV1+/+ mice (Fig. 18a). Survival curve of TRPV1+/+ RTX-pre-treated DSS drinking animals showed significant lower survival probability compared to TRPV1+/+ DSS-treated mice (Fig. 18b).

Myeloperoxidase activity of colon samples

Myeloperoxidase (MPO) activity values of proximal, intermediate and distal colon segments were measured separately. DSS treatment induced significant elevation of MPO level in all experimental groups compared to solvent drinking control (Fig. 19a, b, c). TRPV1 knockout animals showed more than two-fold increase of MPO activity compared to TRPV1+/+ mice in the intermediate colon segment (Fig. 19b). 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 compared to TRPV1+/+ animals (Fig. 19b, c).

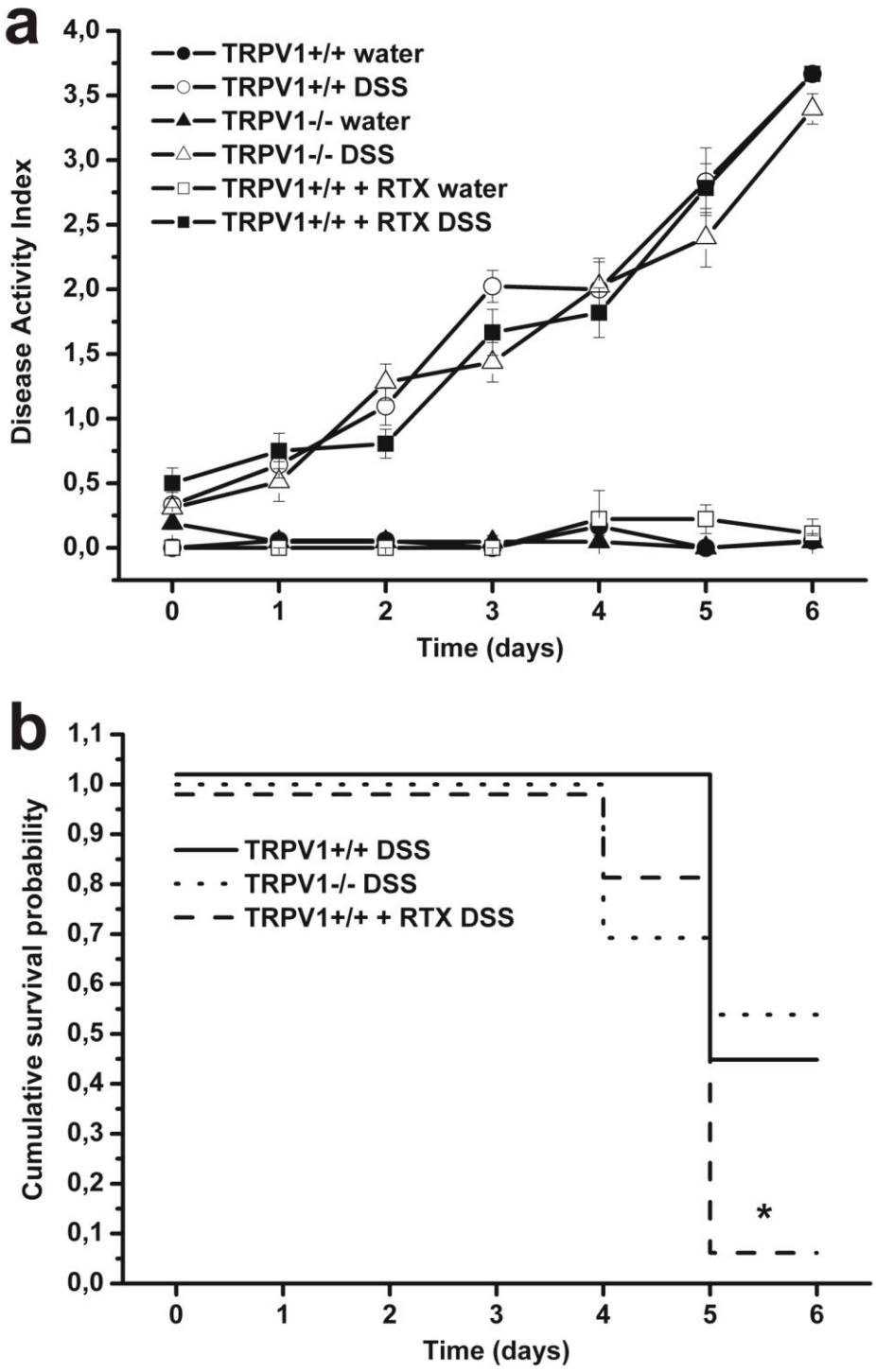

Figure 18. (**a**) Effect of 2% dextran sulphate sodium in drinking water for 6 days on disease activity index of TRPV1+/+, TRPV1-/- and TRPV1+/+ RTX-pre-treated mice. Body weight loss, stool consistency, stool blood content were scored and disease activity index was calculated daily. Comparisons between different groups were made by unpaired t test. Values are means±SEM, n=6-14. (**b**) Survival curves of dextran sulphate sodium-treated TRPV1+/+, TRPV1-/- and TRPV1+/+ RTX-pre-treated animals. Comparisons between different groups were made by logrank test. Values are means±SEM, n=12-14. *P<0.05 vs. TRPV1+/+ mice.

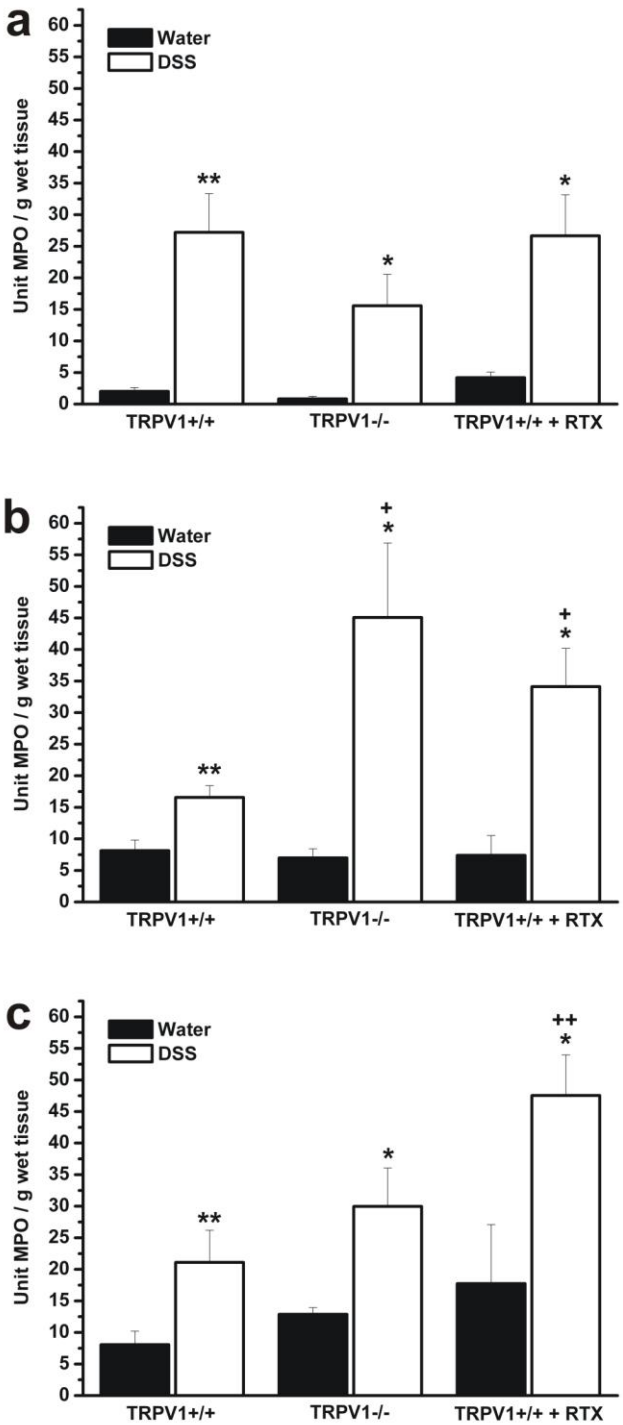

Figure 19. Effect of 2% dextran sulphate sodium (DSS) on neutrophil accumulation in the colon of TRPV1+/+, TRPV1-/- and TRPV1+/+ RTX-pre-treated animals. Measurements were performed after consumption of DSS in drinking water for 6 days (5 days by TRPV1+/+ RTX-pre-treated mice). Myeloperoxidase enzyme (MPO) activity of (**a**) proximal, (**b**) intermediate and (**c**) distal colon segments was determined separately. Data are expressed as MPO content per g wet tissue. Statistical evaluation was performed by unpaired t test. Values are means±SEM, n=6-8. *P<0.05, **P<0.01 vs. distilled water. +P<0.05, ++P<0.01 vs. DSS treated TRPV1+/+ animals.

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 (Fig. 20a, b, c). Genetic lack of the TRPV1 receptor induced significantly more enhanced histological changes compared to wild-type mice in the intermediate and distal colon segments (Fig. 20b, c). RTX pre-treatment, which depletes sensory neuropeptide content of capsaicin-sensitive nerve endings, resulted in significantly more pronounced inflammatory processes compared to TRPV1+/+ mice (Fig. 20b, c). Representative light micrographs illustrate histopathological features (Fig. 21).

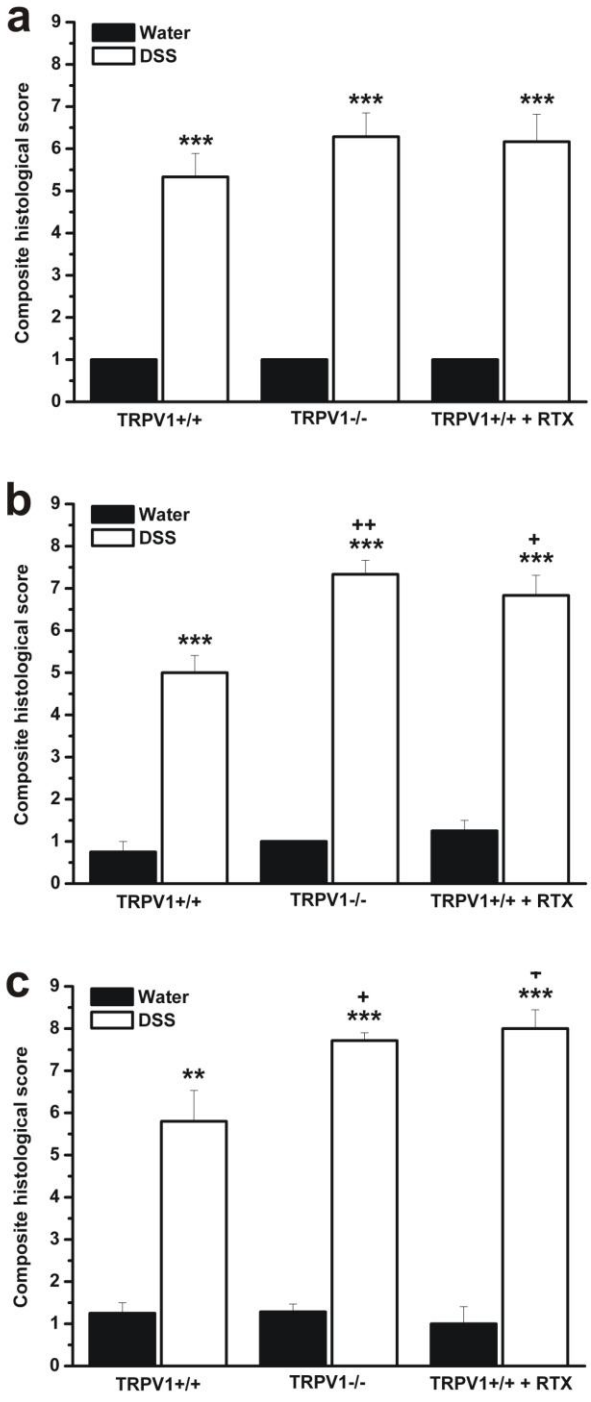

Figure 20. Histological changes in the colons of TRPV1+/+, TRPV1-/- and TRPV1+/+ RTX-pre-treated mice after drinking 2% dextran sulphate sodium (DSS) in water for 6 or 5 (TRPV1+/+ RTX-pre-treated animals) days. Severity of cell accumulation, extent of inflammation, degree of crypt damage was scored and composite histological score was calculated. Histological features of (**a**) proximal, (**b**) intermediate and (**c**) distal colon segments were evaluated separately. Statistical evaluation was performed by ANOVA followed by Bonferroni's test. Values are means±SEM, n=6-8. **P<0.01, ***P<0.001 vs. water drinking control. +P<0.05, ++P<0.01 vs. DSS-treated TRPV1+/+ mice.

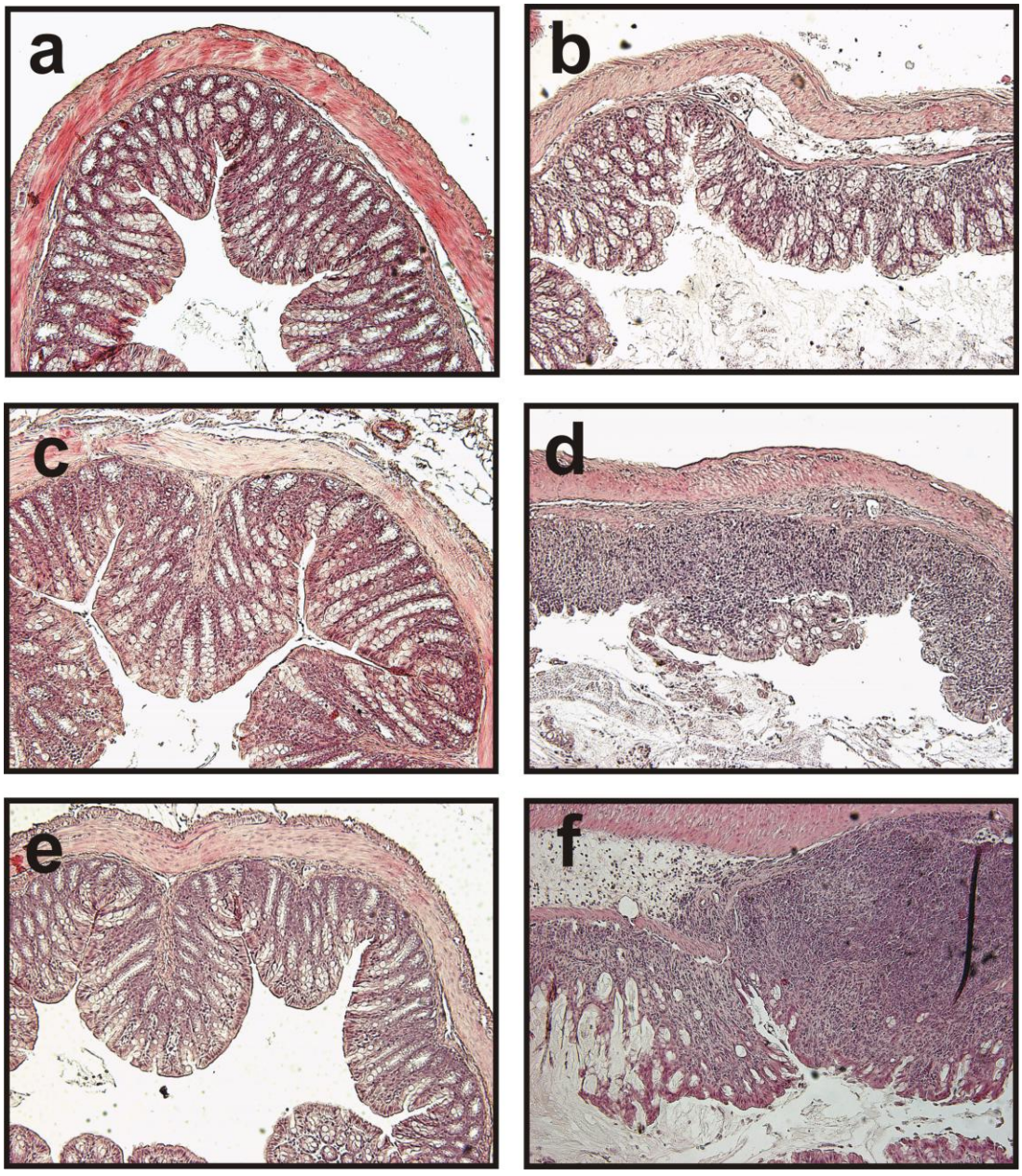

Figure 21. Representative light micrographs (100 X). Intermediate colon segment of water (**a**) and 2% dextran sulphate sodium (DSS) (**b**) treated TRPV1+/+ animal; water (**c**) and 2% DSS (**d**) treated TRPV1-/- animal; water (**e**) and 2% DSS (**f**) drinking TRPV1+/+ RTX-pre-treated animal.

Discussion

Major findings of the present study are that genetic lack of TRPV1 receptors as well as desensitization of the capsaicin-sensitive sensory nerve endings aggravate histopathological changes and increase the number of accumulated neutrophils in dextran sulphate sodium-induced experimental colitis in C57BL/6 mice. These alterations can be observed in the intermediate and distal colon segments. Inflammation of the proximal segment does not show significant differences in TRPV1 receptor knockout or RTX-pre-treated desensitized animals compared to wild-type controls. The role of neurogenic modulatory component in the pathogenesis of inflammatory bowel diseases has been raised for more than ten years ago (Gross & Pothoulakis, 2007). Sensory neuropeptides, such as substance P (SP), corticotrophin-releasing hormone, neurotensin, vasoactive intestinal peptide, mu-opioid receptor agonists and galanin may play a potentially key role in inflammatory bowel disease. Several lines of evidence implicate that elevated SP level correlates with disease activity in certain experimental colitis model (Reinshagen et al., 1997). Reinshagen and co-workers demonstrated that sensory denervation with the neurotoxin capsaicin worsened the inflammation in an acute and chronic model of experimental colitis which suggests a protective role of sensory fibres during gut inflammation (Reinshagen et al., 1998). They found that the CGRP antagonist CGRP-8-37 and immunoneutralising CGRP antibody increased the severity of inflammation in an immune-complex model of colitis. According to these findings they suggested a protective role of CGRP during chronic experimental colitis. Corticotropin-releasing hormone receptor 2-deficient mice showed reduced intestinal inflammatory responses (Kokkotou et al., 2006). Neurotensin stimulates intestinal wound healing following chronic inflammation (Brun et al., 2005). Antagonism of VIP returned jejunal fluid absorption to normal values in iodoacetamide-evoked colitis in rats (Mourad et al., 2006). Chronic administration of galanin attenuates TNBS-induced colitis in rats (Talero et al., 2007). Philippe and co-workers suggest beneficial effects of μ opioid agonists in inflammatory bowel disease (Philippe et al., 2006).

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, such as chronic relapsing diarrhoea, rectal bleeding and inflammation, mucosal ulceration and microscopic crypt abscesses. Ulcerated areas demonstrate markedly increased epithelial cell apoptosis and necrosis by histologic examination (Boismenu et al., 2002; Vetuschi et al., 2002).

Data about the 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. They report that TRPV1 antagonists (capsazepine, JNJ 10185734) and functional denervation of capsaicin-sensitive fibres by neonatal capsaicin treatment decrease the severity of colitis in rats (Kihara et al., 2003; Kimball et al., 2004). In contrast with 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 that sensory neuropeptides exert an anti-inflammatory cumulative effect 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. Since *in vivo* effectiveness of TRPV1 receptor antagonists is questionable, investigation on TRPV1 receptor knockout animals and wild-type counterparts means the most appropriate experimental approach.

Conflicting results may be explained by strain and site specific susceptibility of the colonic mucosa. Studies show that guinea pigs and C57BL/6 mice are more susceptible to the inflammatory effect of DSS (Iwanaga et al., 1994; Krieglstein et al., 2001). In addition different species and strains show most severe inflammatory reactions in different segments of the colon. Swiss-Webster, CBA/j mice and Fisher 344 rats develop left sided colitis (Okayasu et al., 1990; Cooper et al., 1993; Domek et al., 1995), whereas Wistar rats, hamsters and guinea pigs develop right-sided colitis (Yamada et al., 1992; Tamaru et al., 1993; Iwanaga et al., 1994).

According to earlier studies disease activity index shows an excellent correlation with crypt architectural changes (Cooper et al., 1993). In our experiments disease activity index did not follow the differences of myeloperoxidase activity and histological changes properly. On the other hand, survival analysis proved that the

RTX-pre-treated animals had significantly less probability to survive the 6 days protocol.

In conclusion we suggest that learning more about modulatory role of TRPV1 receptors and capsaicin-sensitive nerve endings in the inflammatory bowel disease 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 using matrix-assisted laser desorption/ionization. The ultra-potent TRPV1 receptor agonist resiniferatoxin proved to be able to induce a twofold increase in the plasma PACAP-38 level. On the other hand, bilateral electrical stimulation of the sciatic nerves was incapable of evoking such an increase.

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 and dose dependently diminished mustard oil-induced neurogenic plasma extravasation in the mouse ear. PACAP-38 given intravenously also inhibited mustard oil-induced inflammation in the rat hindpaw. Neurogenic inflammation of the rat hinpaw evoked by intraplantar administration of capsaicin or resiniferatoxin was significantly diminished by systemic PACAP-38 administration. Intravenous PACAP-38 successfully mitigated carrageenan-induced inflammation of the rat hindpaw, which model involves both neurogenic and non-neurogenic inflammatory mechanisms.

3. We observed sensitizing effect of PMA-induced inflammation on acetone-evoked oedema in the mouse ear. These findings reveal a process where non-neurogenic inflammatory mediators sensitize afferent neurons in distal parts of the body. Our data demonstrate that 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. Genetic lack of TRPV1 receptor did not influence sensitizing effect of PMA-induced inflammation. We found that

prostanoids are important mediators in the process, but IL-1β does not have any important action. Ibuprofen, a cyclooxygenase inhibitor, significantly diminished acetone-induced ear oedema which arises upon the basis of the potentiating effect of PMA ear inflammation. Intravenously applied anti-IL-1β antibody did not inhibit acetone-evoked ear oedema.

4. Our results elucidated some unclear details of the pathomechanism of PMA induced inflammation. We found that in contempt of heavy involvement of capsaicin-sensitive nerve endings in PMA-induced ear inflammation, 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, because anti IL-1β antibody did not attenuate ear swelling in this model.

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.

On the whole our results emphasize anti-inflammatory cumulative action of neuropeptides derived from capsaicin-sensitive nerve terminals in different animal models of inflammatory disease. Our data also accentuate that capsaicin-sensitive neurons can become targets of sensitizing effect of non-neurogenic inflammatory processes. Thriving knowledge about diverse role of capsaicin-sensitive afferents and TRPV1 receptors in inflammatory disease may promote the development of novel, clinically useful anti-inflammatory agents.

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