PHD THESIS

INVESTIGATION OF THE FUNCTION AND PHARMACOLOGY OF CAPSAICIN-SENSITIVE SENSORY NEURONS AND THE CAPSAICIN VR1/TRPV1 RECEPTOR USING *IN VIVO* NOCICEPTIVE TESTS

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INTRODUCTION

Nociceptors are specialized nerve endings which detect potentially harmful stimuli and transmit them to the central nervous system. Cutaneous nociceptive primary afferents can be classified based on their axons as myelinated, fast-conducting Aδ nociceptors and unmyelinated slowly-conducting C nociceptors. Concerning phasic stimuli, the previous are responsible for the fast, sharp component of pain sensation, whereas the latter generate the second, slower, diffuse and blunt component. Based on their sensitivity, a considerable fraction of nociceptors are polymodal i.e. sensitive to noxious heat and mechanical stimuli as well as endogenous and exogenous chemical substances.

1. CAPSAICIN-SENSITIVE NOCICEPTORS

Capsaicin, the pungent compound of chilli pepper has played an outstanding role in the investigation of nociceptive primary afferents as a selective test agent. Capsaicin-sensitive sensory neurons in the skin are polymodal nociceptors which comprise the majority of C and Aδ nociceptors. Capsaicin selectively excites these fibres and at higher concentrations and upon longer exposure activation is followed by a persistent functional blockade. This process results in a decreased responsiveness of the neuron to all stimuli; however, selectivity ensures that other sensory modalities (touch, cold sensitivity, taste etc.) remain intact (Jancsó, 1960; Szolcsányi, 1977).

Orthodromic or antidromic stimulation of capsaicin-sensitive nociceptors elicits vasodilatation and plasma protein extravasation in the innervated area. This phenomenon is denominated neurogenic inflammation (Jancsó et al., 1967; 1968) which is induced by neuropeptides released by exocytosis from the capsaicin-sensitive nerve endings, such as tachykinins (substance P, neurokinin A and B) and calcitonin gene-related peptide (CGRP) (Maggi, 1995). Capsaicin-sensitive nerves have therefore a dual function: their efferent function is the transmission of action potentials from the periphery to the central nervous system, whereas neuropeptides released from the peripheral nerve endings mediate a local effector function (Szolcsányi, 1996). Nerve endings also contain neuropeptides other than tachykinins and CGRP, e.g. somatostatin which is also liberated upon activation (Szolcsányi et al., 1998a,b).

2. THE CAPSAICIN VR1/TRPV1 RECEPTOR

The VR1/TRPV1 receptor identified as capsaicin's receptor was the first heat sensitive ion channel to be discovered (Caterina et al., 1997). Besides capsaicin, noxious heat stimuli (> 43 °C), low pH, other exogenous irritants (e.g. resiniferatoxin) and endogenous mediators (anandamide, lipoxygenase products, N-oleoyl-dopamine) are capable of activating the receptor. It can be considered therefore an integrator molecule of different physical and chemical painful stimuli (Tominaga et al., 1998). When the receptor is activated, the opening of the channel pore leads to an influx of Na^+ and Ca^{2+} ions which depolarize the nerve ending and eventually contribute to action potential formation, and Ca^{2+} ions also induce exocytosis of stored neuropeptides.

3. ROLE OF THE TRPV1 RECEPTOR IN THE SENSITIZATION OF NOCICEPTORS

Tissue injury or inflammation induces hyperalgesia, i.e. enhanced pain sensation which by definition implies that the stimulus intensity–pain sensation curve is shifted to the left and its maximum is increased. A major component of the development of thermal hyperalgesia is the sensitization of the peripheral endings of nociceptive afferents during which their threshold decreases and suprathreshold stimuli evoke larger responses. A possible mechanism of heat sensitization is that sensitivity of the TRPV1 receptor is increased due to phosphorylation. There are more and more available data concerning that mediators capable of sensitizing nociceptors to heat (bradykinin, prostaglandins, ATP, serotonin etc.) lead to enhanced heat responsiveness by activating signal transduction pathways involving protein kinases (protein kinase C – PKC, protein kinase A – PKA) which phosphorylate the TRPV1 receptor (Tominaga et al., 2001; Sugiura et al., 2002; Moriyama et al., 2005). Recent reports show that various lipoxygenase products are able to excite directly the TRPV1 receptor (Hwang et al., 2000). The importance of TRPV1 receptor is further supported by the finding that no inflammatory thermal hyperalgesia was found in mice lacking the receptor (Caterina et al., 2000; Davis et al., 2000). In conclusion, TRPV1 receptor-expressing neurons are potential peripheral targets for the development of new analgesic drugs.

4. ANTI-INFLAMMATORY AND ANTINOCICEPTIVE EFFECT OF SOMATOSTATIN RELEASED FROM CAPSAICIN-SENSITIVE NERVE ENDINGS

Tachykinins and CGRP released from capsaicin-sensitive fibres induce local inflammation in the innervation area, somatostatin, however, enters the circulation and exerts a systemic anti-

inflammatory (Szolcsányi et al., 1998a,b) and antinociceptive effect (Helyes et al., 2000). Besides the afferent and local efferent functions, capsaicin-sensitive neurons have therefore a systemic, neurohormonal regulatory or "sensocrine" function (Thán et al., 2000).

5. CANNABINOID RECEPTORS AND THEIR AGONISTS: NEW TARGETS OF ANALGESIA

The first identified endogenous cannabinoid, anandamide binds mainly to cannabinoid CB_1 receptors which are found in large numbers in the central nervous system in areas of afferent nociceptive pathways and descending inhibitory pathways as well. This indicates that they play a role in the central modulation of pain perception (Pertwee, 2001). The presence of CB_1 receptors was also shown on primary afferents which can mediate a potential peripheral antinociceptive effect of cannabinoids. CB_2 receptor expression was previously described on non-neuronal cells which could be responsible for the immunomodulatory effect of cannabinoids but there are data pointing to the existence of further, CB_2-like receptors which also participate in the control of pain transmission (Calignano et al., 1998).

AIMS

Our experiments aimed at the *in vivo* investigation of potential new targets of analgesia located on TRPV1 receptor-expressing neurons using animal models of nociception and testing new compounds which act on these targets. Our goals were the following:

I. Investigating the **effect of endogenously occurring cannabinoids, anandamide and palmitoyl-ethanolamide** on TRPV1 receptor stimulation-induced **sensory neuropeptide release and on neuropathic mechanical hyperalgesia.**

II. Investigating the **antinociceptive effects of the stable and potent heptapeptide somatostatin receptor agonist TT-232**.

III. Comparing wild-type and **TRPV1 receptor gene deficient mice in acute and chronic nociceptive models** *in vivo.*

IV. Developing a reliable, **new thermonociceptive test based on measurement of the heat threshold temperature.**

I. INHIBITORY EFFECT OF ANANDAMIDE (ANA) AND PALMITOYL-ETHANOLAMIDE (PEA) ON RESINIFERATOXIN-INDUCED SENSORY NEUROPEPTIDE RELEASE *IN VIVO* AND NEUROPATHIC HYPERALGESIA

Anandamide (ANA) was shown to have antinociceptive activity via CB_1 receptor stimulation in various *in vivo* animal models and it also effectively diminished inflammatory heat and mechanical hyperalgesia (Calignano et al., 1998; Jaggar et al., 1998; Richardson et al., 1998). Palmitoyl-ethanolamide (PEA), another endogenous cannabinoid thought to act on a peripheral CB_2-like receptor, was also effective in different nociceptive tests (Calignano et al., 1998, 2001; Jaggar et al., 1998).

Anandamide is also able to activate TRPV1 receptors *in vitro* (Zygmunt et al., 1999; Smart et al., 2000), although the *in vivo* role and relevance of this effect is subject to a debate. The disagreement is based on the fact that CB_1 and TRPV1 receptors are expressed by the same group of small neurons (Ahluwalia et al., 2000) and the concentration needed to excite TRPV1 receptors is orders of magnitude higher than the level which exerts an inhibitory effect on sensory neurons via CB_1 receptors (Szolcsányi, 2000a,b).

METHODS

1. Measurement of resiniferatoxin-induced CGRP and somatostatin release *in vivo:* In anaesthetized rats resiniferatoxin was injected (RTX, 0.6 μg/kg i.v.) to evoke neuropeptide release which was measured from arterial blood samples collected 5 min after injection. Animals were pretreated with different doses of ANA or PEA (10 or 100 μg/kg i.v.) and CB_1 or CB_2 receptor antagonists were applied (SR141716A or SR144528, 100 μg/kg i.v.) 10 min before the respective cannabinoid treatment. CGRP and somatostatin concentrations were determined from the plasma by sensitive radioimmunoassay (RIA) methods.

2. Partial sciatic nerve ligation-induced (traumatic) neuropathic mechanical hyperalgesia (Seltzer-model): The mechanonociceptive threshold of rats was measured with the Randall-Selitto test. The animal's hind paw was inserted between the cone-shaped pushers of the analgesimeter equipment which exerted a continuously increasing force on the limb. The force at which the animal withdrew its paw was considered as mechanonociceptive threshold. Under anaesthesia, 1/3-1/2 part of the sciatic nerve was ligated unilaterally. One week later the effect of ANA or PEA (100 μg/kg i.p.) on the developed hyperalgesia was

investigated. CB_1 and/or CB_2 receptor antagonist (SR141716A or SR144528, 3 mg/kg i.p.) was administered 30 min prior to ANA or PEA treatment.

RESULTS

1. Effect of ANA and PEA on plasma CGRP and somatostatin concentrations: RTX (0.1-3 μg/kg i.v.) dose-dependently increased plasma CGRP and somatostatin levels. Basal concentrations of neuropeptides were influenced neither by ANA, nor by PEA, however, RTX-induced CGRP and somatostatin release was dose-dependently diminished by ANA which was inhibited by pretreatment with the CB_1 receptor antagonist SR141617A (100 μg/kg i.v.). Likewise, PEA dose-dependently decreased sensory neuropeptide release evoked by RTX injection.

2. Effect of ANA and PEA on neuropathic mechanical hyperalgesia following partial sciatic nerve lesion: Seven days following partial ligation of the sciatic nerve, mechanonociceptive threshold of the animals decreased by 29.7 ± 0.6%. ANA treatment (100 μg/kg i.p.) completely abolished hyperalgesia. Pretreatment with the CB_1 receptor antagonist SR141617A (3 mg/kg i.p.) by itself enhanced hyperalgesia by 37.1% and totally inhibited the antihyperalgesic action of subsequent ANA injection. PEA (100 μg/kg i.p.) diminished hyperalgesia by 79.4% and this effect was prevented by the CB_2 receptor antagonist SR144528 (3 mg/kg i.p.). Similarly to the CB_1 receptor antagonist, this compound also increased the threshold drop by 47.5%. Combination of the two antagonists, however, did not produce an additive effect on the aggravation of hyperalgesia.

CONCLUSIONS

Our results have demonstrated that both anandamide (ANA) and palmitoyl-ethanolamide (PEA) inhibited the sensory neuropeptide release *in vivo* induced by injection of the TRPV1 receptor agonist RTX, via CB_1 and CB_2-like receptors, respectively, while they failed to influence basal plasma CGRP and somatostatin levels. The potential TRPV1 receptor-activating effect of ANA is likely to be counteracted by that it binds and activates CB_1 receptors on the same nerve endings with higher affinity.

Both cannabinoid agonists effectively decreased traumatic neuropathic mechanical hyperalgesia, likewise by activating CB_1 and CB_2-like receptors, respectively. One possible mechanism of this effect is that they inhibit sensory neuropeptide release from capsaicin-sensitive primary afferents. Antagonists of the CB_1 and CB_2 receptors alone or in combination

aggravated mechanical hyperalgesia which indicates that endocannabinoids exert a tonic inhibitory effect in neuropathy which alleviates hyperalgesia.

In conclusion, cannabinoid receptor agonists – especially selective CB_2 receptor agonists lacking central effects – offer a new therapeutic possibility for the treatment of neuropathic pain.

II. ANALGESIC EFFECT OF TT-232, A HEPTAPEPTIDE SOMATOSTATIN ANALOGUE, IN ACUTE PAIN MODELS OF THE RAT AND THE MOUSE AND IN STREPTOZOTOCIN-INDUCED DIABETIC MECHANICAL ALLODYNIA

The systemic anti-inflammatory and antinociceptive effect of somatostatin released upon activation of capsaicin-sensitive nerves makes possible the development of novel, peripherally-acting anti-inflammatory and analgesic drugs. Native somatostatin is not suitable for this therapeutic use because due to its widespread physiological roles it affects several endocrine and gastrointestinal functions in the body and furthermore, its plasma half-life is very short ($T_{1/2}$= 3 min).

Among the five somatostatin receptor subtypes (sst_{1-5}) sst_1 and sst_4 receptors mediate no endocrine effects, however, they are expressed by sensory neurons which makes them therefore potential selective targets. In our department investigation of stable somatostatin analogues lacking endocrine side effects has recently been started. The heptapeptide TT-232 (D-Phe-Cys-Tyr-D-Trp-Lys-Cys-Thr-NH_2) synthesized by the Peptide-Biochemistry Research Group of the Hungarian Academy of Sciences – which otherwise has a potent antiproliferative effect – did not influence growth hormone and gastrine secretion (Kéri et al., 1996). TT-232 mainly binds to sst_4 receptors (Helyes et al., 2005). It effectively decreased sensory neuropeptide release *in vivo* and potently inhibited neurogenic and non-neurogenic inflammation in various models (Helyes et al., 2001; Pintér et al., 2002). It diminished Complete Freund's Adjuvant (CFA)-induced joint swelling and mechanical hyperalgesia (Helyes et al., 2004) and traumatic neuropathic hyperalgesia as well (Pintér et al., 2002).

METHODS

1. Formalin test: On rats, nocifensive reaction was evoked by intraplantar injection of formalin (2.5%, 50 μl i.pl.) which appears in two phases: the first phase lasting from 0-5 min and the second from 20-45 min after injection. Various doses of TT-232 (20-80 μg/kg) were

administered intraperitoneally (0.1 ml/100 g i.p.) 30 min before formalin injection and the effect of the drug was compared to a solvent-treated group. Quantitative evaluation of the spontaneous nocifensive behaviour was made by the following formula: (2x duration of paw lickings + 1x duration of paw liftings)/observation period (Composite Pain Score – CPS).

2. Phenylquinone-induced abdominal constriction ("writhing") test: Abdominal constrictions were induced by intraperitoneal phenylquinone injection (0.02%, 0.2 ml) which is considered a model of visceral nociception. TT-232 was administered subcutaneously 30 min before (5-200 μg/kg s.c.). The number of abdominal constrictions was counted in a 20-min period following phenylquinone injection and results were compared to a solvent-treated group.

3. Measurement of the noxious heat threshold and resiniferatoxin-induced thermal hyperalgesia: The noxious heat threshold of rats was determined by an increasing-temperature hot plate. The animal was placed on the metal plate with a built-in heating unit which was heated up afterwards at an even rate from room temperature until the rat showed nocifensive reaction and the corresponding plate temperature was regarded as heat threshold. After control measurements, animals were treated with TT-232 (10-200 μg/kg i.p.), measurements were repeated 30 min later and results were compared to the initial control thresholds. In another series of experiments, thermal hyperalgesia was evoked by intraplantar resiniferatoxin (RTX, 0.05 nmol i.pl.) and threshold determinations were repeated 5, 10, 15 and 20 min after injection. Different doses of TT-232 (5-100 μg/kg i.p.) were administered 10 min prior to RTX. On each occasion, half of the group was treated with the vehicle which allowed comparisons to an actual solvent control.

4. Measurement of diabetic neuropathic mechanical allodynia: Experimental diabetes mellitus was generated by 50 mg/kg i.v. streptozotocin in rats. Two weeks later, blood glucose levels were measured from samples taken from the tail vein with an Accu-Check glucometer (Roche) and only animals with a level higher than 15 mmol/l were included in the further studies. Mechanonociceptive thresholds of freely moving rats were determined by a dynamic plantar aesthesiometer (Ugo Basile). This equipment has a blunt needle which is pushed to the plantar surface of the paw with a continuously increasing force at a preset rate until the animal withdraws its paw. At this point the needle falls to its initial position and mechanonociceptive threshold is read on the display. After diabetes was established, thresholds were measured weekly until mechanical allodynia developed. Animals were treated

then with various doses of TT-232 (2.5-100 μg/kg i.p.) and threshold measurements were repeated 30 min later.

RESULTS

1. Effect of TT-232 on formalin-evoked nocifensive reactions: The first phase of formalin-evoked nocifensive reaction was only inhibited significantly by the 80 μg/kg i.p. dose based on the evaluation of CPS. In the second phase TT-232 showed a bell-shaped dose-response curve, as the doses of 40 and 80 μg/kg i.p. had significant antinociceptive effect but 160 μg/kg failed to decrease CPS. Diclofenac used as a reference drug only inhibited nocifensive behaviour in the second phase at a dose of 50 mg/kg i.p.

2. Effect of TT-232 on phenylquinone-evoked abdominal constrictions: TT-232-pretreatment (10-200 μg/kg s.c.) significantly diminished the number of writhing movements induced by i.p. phenylquinone injection, however, no dose-response relationship could be established. Doses of 20 and 200 μg/kg produced the maximum inhibition (70 and 75% percentage inhibition), while the effect of doses in between resulted in a bell-shaped dose-response curve, similarly to that observed in the formalin test.

3. Effect of TT-232 on the noxious heat threshold and on resiniferatoxin-induced thermal hyperalgesia: Control heat threshold of rats was 44.5 ± 0.2 °C. TT-232 significantly increased the heat threshold at a dose range of 20-200 μg/kg, a clear-cut dose-dependent relationship was not seen in this test either. The maximal increase (1.48 ± 0.4 °C) was produced by the 200 μg/kg i.p. dose.

Intraplantar resiniferatoxin evoked a 7.39 ± 1.3 °C drop of heat threshold 5 min after injection. Pretreatment with TT-232 significantly decreased thermal hyperalgesia at doses between 10-50 μg/kg i.p., the effect of a higher, 100 μg/kg dose, however, was not significant.

4. Effect of TT-232 on diabetic neuropathic mechanical allodynia: Mechanonociceptive threshold decreased by 28.6 ± 3.1% 5 weeks after streptozotocin treatment. TT-232 significantly diminished mechanical allodynia at doses of 10, 20 and 100 μg/kg i.p., among which 20 μg/kg exerted the maximal, 54% inhibition. The mechanical threshold of naïve rats was not influenced by 20 μg/kg i.p. TT-232.

CONCLUSIONS

Our results have demonstrated that the peripherally-acting somatostatin receptor agonist TT-232 had a pronounced analgesic effect in nociceptive processes of various origins, in rats and mice as well. The effects of low doses of the compound were detectable in the conventionally used chemonociceptive tests, the novel thermonociceptive test and the diabetic polyneuropathy model. In the formalin test TT-232 proved to be approximately 1000 times more potent than diclofenac, while in the thermonociceptive tests – compared to our previous results (Almási et al., 2003) – it was 300 times more potent than morphine or diclofenac. The advantage of TT-232 is that due to its selective target of action it lacks the side effects which could appear as a consequence of somatostatin's widespread actions. Unwanted effects are also reduced by the fact that it does not penetrate the blood-brain barrier. TT-232 is a promising candidate to be a novel analgesic drug with a broad profile which includes therapy-resistant neuropathic conditions.

III. INVESTIGATION OF THE ROLE OF TRPV1 RECEPTORS IN ACUTE AND CHRONIC NOCICEPTIVE PROCESSES USING GENE-DEFICIENT MICE

The investigation of the roles of TRPV1 receptor-expressing polymodal nociceptors started with experiments on capsaicin's selective excitatory and consequent blocking effects. Examining the desensitizing action of capsaicin, however, only provides information on the functions of the whole fibre but not the receptor itself.

The use of the majority of receptor antagonists (capsazepine, ruthenium red, iodo-resiniferatoxin – I-RTX) can be impaired by problems with selectivity (Docherty et al., 1997; Liu & Simon, 1997) and *in vivo* efficacy (Jakab et al., 2005) and furthermore I-RTX may potentially act as an agonist if it is converted into RTX in the body.

Cloning the TRPV1 receptor opened the way to the generation and *in vivo* investigation of gene-deleted (knockout) mice (Davis et al., 2000; Caterina et al., 2000). Results showed that heat sensitivity of untreated knockout mice did not differ from the wild-type counterparts which was surprising given that capsaicin-desensitized animals had higher heat thresholds (Szolcsányi, 1985; Szolcsányi, 1987). However, inflammatory thermal hyperalgesia did not develop in the absence of the receptor which indicates that heat sensitization of nociceptors requires the TRPV1 receptor.

METHODS

Animals: In these experiments TRPV1 receptor gene deficient ($TRPV1^{-/-}$) and wild-type mice ($TRPV1^{+/+}$) were used.

1. Phorbolesther-induced acute chemonociception (PMA test): Intraplantar injection of the protein kinase C (PKC) activator phorbolesther, phorbol 12-myristate 13-acetate (PMA, 10 μg/ml, 20 μl) was used to induce nocifensive behaviour which was observed during 45 min after injection. For quantitative evaluation of the test, the time spent licking and lifting of the paw was measured.

2. Formalin test: Intraplantar injection of formalin (2.5%, 20 μl i.pl.) was used to induce nocifensive behaviour which appears in two phases: the first phase lasting from 0-5 min and the second from 20-45 min after injection. For quantitative evaluation of the test, the time spent licking and lifting of the paw was measured.

3. Heat injury-induced thermal and mechanical hyperalgesia: Noxious heat threshold of mice was measured by an increasing-temperature hot plate and in another group of animals mechanonociceptive threshold was determined by a dynamic plantar aesthesiometer (Ugo Basile). After control measurements, under ether anaesthesia one of the hind paws was immersed into a 51 °C water bath for 15 sec and measurements were repeated afterwards.

4. Inflammatory mechanical hyperalgesia evoked by intraplantar carrageenan: Mechanonociceptive thresholds of mice were determined by the dynamic plantar aesthesiometer. Intraplantar injection of carrageenan (3%, 100 μl) was applied to induce inflammation of one of the hind limbs. Thresholds were measured again 3 hours later.

5. Streptozotocin-induced diabetic polyneuropathy: Experimental diabetes mellitus was induced by streptozotocin treatment (STZ, 250 mg/kg i.v.). 2 weeks later, blood glucose concentrations were measured from samples drawn from the tail vein with an Accu-Check glucometer (Roche) and further investigations were only performed on animals with blood glucose levels higher than 15 mmol/l. Mechanonociceptive thresholds of mice were determined by aesthesiometry.

6. Cisplatin-induced toxic neuropathy: Mice were treated with cisplatin three times a week during 5 weeks (2 mg/kg i.p., cumulative dose 30 mg/kg). Mechanonociceptive thresholds were measured by aesthesiometry.

7. Traumatic mononeuropathy induced by partial sciatic nerve lesion: Under anaesthesia 1/3-1/2 part of the sciatic nerve of mice was ligated unilaterally. Mechanonociceptive thresholds were measured by the dynamic plantar aesthesiometer.

8. Measurement of plasma somatostatin concentrations in the chronic polyneuropathy models: Animals were fastened during a night to ascertain that gastrointestinal somatostatin release was minimal. Under anaesthesia arterial blood samples were collected and somatostatin concentrations extracted from the plasma were determined by a radioimmunoassay (RIA) developed in our department (Németh et al., 1996). The time of sampling was chosen to correspond to the period when maximal differences had been found between the behavioural thresholds of the two groups of mice.

RESULTS

1. PMA test: In wild-type mice ($TRPV1^{+/+}$) PMA induced an acute nocifensive reaction (paw licking and lifting) which lasted from 5-45 min after injection. The total duration of paw lickings and liftings was 669.2 ± 170.8 sec. Mice lacking the TRPV1 receptor ($TRPV1^{-/-}$) did not respond to PMA as the duration of the nocifensive reaction did not differ from that evoked by the solvent (16.8 ± 8 sec and 20.2 ± 10.3 sec).

2. Formalin test: Intraplantar injection of formalin induced a two-phase nocifensive reaction The total duration of paw lickings and liftings of $TRPV1^{+/+}$ and $TRPV1^{-/-}$ mice in the first phase (0-5 min) were 130.7 ± 12.6 sec and 99.7 ± 16.1 sec, in the second phase (20-45 min) 268.7 ± 50.7 sec and 363.6.6 ± 37.8 sec, respectively. Statistical analysis revealed no significant difference between the two groups in either phase.

3. Heat injury-induced thermal and mechanical hyperalgesia: Heat thresholds of untreated $TRPV1^{+/+}$ and $TRPV1^{-/-}$ mice were 44.3 ± 0.4 °C and 44.4 ± 0.3 °C, while their mechanonociceptive thresholds were 7.9 ± 0.3 g and 7.5 ± 0.3 g, respectively. There was no significant difference therefore between the control values of the two groups. The animals recovered from ether anaesthesia within a few minutes after heat injury and showed no signs of spontaneous pain. Decreases in the heat and mechanical thresholds were developed 10 and 20 min following heat injury. Both types of hyperalgesia proved to be significantly reduced in $TRPV1^{-/-}$ mice at each measurement point. The maximal drops of heat threshold were 10.23 ± 1.0 °C and 3.59 ± 0.6 °C, whereas the severest mechanical hyperalgesia values were 56.9 ± 2.4% and 23.6 ± 7.9% in $TRPV1^{+/+}$ and $TRPV1^{-/-}$ mice, respectively.

4. Inflammatory mechanical hyperalgesia evoked by intraplantar carrageenan: The control mechanonociceptive thresholds were 7.85 ± 0.2 g in wild-type and 7.31 ± 0.3 g in TRPV1 receptor knockout mice. Carrageenan injection resulted in the inflammation of the treated limb with visible oedema and redness. 3 hours after treatment mechanical thresholds decreased: in $TRPV1^{+/+}$ mice the threshold dropped to 5.35 ± 0.3 g (31.7 ± 4.1%

hyperalgesia), and in TRPV1$^{-/-}$ animals it decreased to 4.9 ± 0.3 g (31.8 ± 6.1% hyperalgesia). Therefore, no significant difference was found between the two groups.

5. Mechanical hyperalgesia in streptozotocin-induced diabetic polyneuropathy: Control mechanonociceptive threshold of TRPV1$^{+/+}$ mice was 6.7 ± 0.2 g, while in the TRPV1$^{-/-}$ group it was 6.9 ± 0.3 g. Two weeks following STZ treatment experimental diabetes mellitus was present in all mice. In mice lacking the TRPV1 receptor hyperalgesia developed already by the 3rd week after treatment and remained significantly more severe compared to wild-type mice throughout the whole experimental period lasting until the 7th week. The highest difference between the two groups were found on the 5th week (10.29 ± 2.6% hyperalgesia in the TRPV1$^{+/+}$ group and 31.12 ± 2.7% in the TRPV1$^{-/-}$ group).

6. Mechanical hyperalgesia in cisplatin-induced toxic neuropathy: Mechanonociceptive thresholds of cisplatin-treated mice did not change significantly in the first 3 weeks of administration (6.6 ± 0.2 g in both groups). In TRPV1$^{-/-}$ mice significant hyperalgesia started to develop from the 4th week while in wild-type mice only 4 weeks later. From the 8th week, however, there was no significant difference between the two groups. The maximal difference was measured on the 7th week (2.64 ± 4.0% hyperalgesia in the TRPV1$^{+/+}$ group and 7.87 ± 3.6% in the TRPV1$^{-/-}$ group).

7. Mechanical hyperalgesia in traumatic neuropathy: One week after partial sciatic nerve lesion mechanical hyperalgesia developed on the operated limb which was maintained throughout the 5-week experimental period. The highest values were measured on the 2nd week when hyperalgesia was 45.13 ± 4.7% in TRPV1$^{+/+}$ and 40.53 ± 4.0% in TRPV1$^{-/-}$ animals. No significant differences were found between wild-type and gene-deleted mice at any measurement points.

8. Plasma somatostatin concentrations in the chronic polyneuropathy models: Plasma somatostatin concentrations of naïve mice were 8.5 ± 0.2 fmol/ml in the TRPV1$^{+/+}$ group and 7.44 ± 0.6 fmol/ml in TRPV1$^{-/-}$ mice. Blood sampling of neuropathic mice was performed 5 weeks after STZ injection and 7 weeks following the beginning of cisplatin treatment, when maximal differences were observed between the mechanical hyperalgesia of the two groups. Plasma somatostatin levels of neuropathic TRPV1$^{+/+}$ mice were significantly elevated compared to naïve mice, both in diabetic (10.08 ± 0.6 fmol/ml) and cisplatin-treated animals (10.46 ± 0.9 fmol/ml). On the contrary, no increase was found in TRPV1$^{-/-}$ mice, plasma somatostatin levels were 8.02 ± 0.6 fmol/ml and 7.63 ± 0.5 fmol/ml, respectively.

CONCLUSIONS

With the investigation of TRPV1 receptor gene deficient mice we have demonstrated that this noxious stimulus-gated ion channel is essential in phorbolesther-induced acute chemonociception and in heat and mechanical hyperalgesia developed after mild heat injury. Neither formalin-induced nocifensive behaviour, nor mechanical hyperalgesia in carrageenan-inflammation or traumatic mononeuropathy were influenced by the lack of TRPV1 receptor. In chronic diabetic and toxic polyneuropathy it had a protective role as mechanical hyperalgesia was less severe and developed later in its presence. There are several pieces of evidence concerning that somatostatin released from capsaicin-sensitive neurons has systemic anti-inflammatory and antinociceptive effect (Szolcsányi et al., 1998a,b; Helyes et al., 2000; Carlton et al., 2001a,b; 2003; Helyes et al., 2004). Our hypothesis was that in polyneuropathic conditions the somatostatin-mediated counter-regulatory mechanism is activated by TRPV1 stimulation and its absence leads to the earlier onset and increase in the severity of hyperalgesia. This could be confirmed by the results of plasma somatostatin concentrations which showed that in wild-type mice somatostatin was increased in polyneuropathic compared to untreated controls, while in TRPV1$^{-/-}$ mice the concentration of the peptide remained unaltered.

In conclusion, in certain models TRPV1 receptor promotes nociception but surprisingly, in chronic polyneuropathy models it mediates an opposite, antinociceptive effect, possibly by the release of somatostatin. In the models where no difference was found in the behaviour of TRPV$^{-/-}$ mice, the receptor is not likely to play a key role, but it is also possible that the two opposing effects extinguish each other. As two contrary functions of TRPV1 receptor were revealed in the investigated models, it is concluded that both antagonists and agonists may have therapeutic value depending on the pathomechanism of the given condition.

IV. DEVELOPMENT OF A HEAT INJURY-INDUCED THERMAL HYPERALGESIA MODEL EMPLOYING A NOVEL INCREASING-TEMPERATURE WATER BATH

Conventional tests of thermonociception are based on exposing the animal's paw or tail to a heat stimulus of constant, suprathreshold intensity, e.g. by placing it on a hot metal surface (constant temperature hot plate) or stimulating it with a focused beam of light (Hargreaves' plantar test), and the time until the appearance of a nocifensive reaction is determined (Le Bars et al., 2001). This latency is considered, not quite consequently, as noxious heat

threshold. The disadvantage of these methods is that upon repeated measurements latency may decrease or increase due to sensitization or habituation and it is not less important that only the effect of opioid analgesics can be reliably detected. A further drawback is that latency values are difficult to compare to heat thresholds routinely determined in electrophysiological experiments (e.g. patch clamp, single fibre recordings).

Applying an increasing heat stimulus, the noxious heat threshold temperature of animals can be measured i.e. the lowest temperature which evokes a nocifensive reaction. Our research group has successfully implemented this measurement principle by developing an increasing-temperature hot plate and a new hyperalgesia model (Almási et al., 2003) which was suitable to detect the antinociceptive and antihyperalgesic effect of low doses of morphine, diclofenac and paracetamol. Besides its excellent pharmacological sensitivity, threshold measurement also complies better with the international ethical guidelines (Zimmermann, 1983), as animals are exposed to the least and shortest possible painful stimuli. In the present experiments another newly developed equipment, the increasing-temperature water bath was used.

METHODS

1. Determination of the noxious heat threshold with the increasing-temperature water bath: The increasing-temperature water bath was developed in cooperation with Experimetria Ltd. (Budapest). The equipment consists of a water container with a built-in heating unit and a separate controlling unit for setting different heating rates and starting temperatures which also has a display showing the actual temperature of the water bath. Rats were lightly restrained and held in an upright position above the water bath allowing free movement of the hind limbs, then one of the hind paws was immersed into the water and the heating process was started afterwards. A starting temperature of 30 °C and a heating rate of 24 °C/min were employed at each measurement. At the moment when the animal withdrew its paw the heating was immediately stopped and the corresponding temperature was recorded as the noxious heat threshold of the examined paw.

2. Induction of thermal hyperalgesia by mild heat injury and assessment of the antihyperalgesic effect of analgesics: Following control measurements, under ether anaesthesia one of the hind paws was immersed in a 51 °C hot water bath for 20 seconds. After recovering from anaesthesia, heat threshold determinations were repeated 10 and 20 minutes after heat injury to confirm the development of hyperalgesia. Drugs were administered after the 20-minute measurement which was followed by repeated measurements at 10 minute intervals. In each measurement series, one half of the group was

injected with the solvent and drug effects were assessed compared to the solvent-treated group.

RESULTS

The noxious heat threshold of untreated rats was 43.1 ± 0.4 °C and was reproducible upon measurements at intervals of 10 minutes which means that no significant difference was found between thresholds of the same paw measured at different time points. Upon heat threshold measurements 10 and 20 min following heat injury, a 7–8 °C drop of the threshold was observed and this heat hyperalgesia was maintained at an even level for at least an hour. Morphine, non selective cyclooxygenase inhibitors diclofenac and ibuprofen or centrally-acting paracetamol administered after the 20-min measurement all dose-dependently reduced the heat injury-induced drop of heat threshold (minimal effective doses (MED): 0.3; 0.3; 10; 30 mg/kg i.p.). The model was suitable for showing the effect of the peripherally-acting somatostatin receptor agonist TT-232 (MED: 0.1 mg/kg i.p). Intraplantar injection of morphine (10 μg), diclofenac (10 μg) and ibuprofen (100 μg) administered 20 minutes after heat injury all significantly decreased subsequent thermal hyperalgesia Lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA, 10 mg/kg i.p.) failed to influence thermal hyperalgesia, bradikinin B_2 receptor antagonist HOE140 (0.1 mg/kg i.p.), however, had a significant inhibitory effect, while the TRPV1 receptor antagonist JYL1421 (2 mg/kg i.p.) almost completely abolished the drop of heat threshold.

CONCLUSIONS

The increasing-temperature water bath was suitable for the reliable and reproducible measurement of the noxious heat threshold of conscious rats. Heat injury, as a naturally occurring noxious impact was used to induce hyperalgesia, in other words, a first degree burn was modelled which led to a pronounced drop of heat threshold. When examining drug effects, the common clinical practice was followed by administrating the compounds after the injury, contrary to typical experimental protocols where pretreatments are performed. The model proved to be remarkably sensitive to the antihyperalgesic effect of both morphine and cyclooxygenase inhibitors and besides the conventional analgesics, it was suitable to show the effect of a compound of a novel target of action, the somatostatin receptor agonist TT-232 (see chapter II). Human doses calculated on the basis of the minimal effective doses obtained with our model are in the range of the recommended doses applied in the clinical practice. The model was also able to detect the effect of locally applied analgesics.

Investigating the pathomechanism of heat injury-induced thermal hyperalgesia we have concluded that prostaglandins definitely played an important role as cyclooxygenase inhibitors inhibited hyperalgesia at low doses and after local treatment as well. We have proven that bradikinin had a major part in the development of heat injury-induced drop of heat threshold and furthermore, activation and sensitization of the TRPV1 receptor was essential in this thermal hyperalgesia. The latter finding is also supported by our results gained with TRPV1 receptor gene-deleted mice (see chapter III). Lipoxygenase inhibition proved to be ineffective in our model which means that lipoxygenase products are either not formed in tissue injury following mild burns or their effect is minor so that its abolishment does not have an impact on hyperalgesia.

In conclusion, the thermonociceptive test developed and validated in our laboratories is a reliable, easily performed and sensitive new method which is suitable for the investigation of peripherally and centrally-acting analgesics. At the same time, heat injury-induced drop of heat threshold is an excellent in vivo model for the examination of the pathomechanism of thermal hyperalgesia.

SUMMARY OF THE NEW FINDINGS PRESENTED IN THE THESIS

1. Our results have proven that **anandamide and palmitoyl-ethanolamide – naturally occurring CB_1 and CB_2 receptor agonists – decreased sensory neuropeptide release *in vivo* induced by the TRPV1 receptor agonist resiniferatoxin.** We have shown that **anandamide and palmitoyl-ethanolamide diminished partial sciatic nerve lesion-induced mechanical hyperalgesia** as well, via CB_1 and CB_2 receptor activation**.** CB_1 and/or CB_2 receptor antagonists enhanced mechanical hyperalgesia *in vivo*, therefore it is suggested that **endogenous cannabinoids exert a tonic antinociceptive effect** in traumatic mechanical hyperalgesia.

2. We have demonstrated that the **somatostatin receptor agonist TT-232 is a potent antinociceptive and antihyperalgesic compound** in acute chemical and thermal nociceptive models and chronic diabetic polyneuropathy.

3. Using gene-deleted mice it has been shown that **in certain acute pain models the presence of TRPV1 receptor was indispensable while in others it had no exclusive role, however, in chronic polyneuropathy models the absence of the receptor surprisingly enhanced mechanical hyperalgesia.** We have hypothesized that as a consequence of **chronic stimulation of TRPV1 receptor counter-regulatory processes could be initiated mediated by systemic action of somatostatin released from TRPV1 receptor-expressing nerve endings.** The TRPV1 receptor may mediate pronociceptive and antinociceptive effects depending on the pathophysiological process, therefore **both agonists and antagonists may be drug candidates.**

4. We have elaborated **a new thermonociceptive test, the heat injury-induced thermal hyperalgesia model based on a self-developed method measuring the noxious heat threshold, which proved to be a very reliable and sensitive method for detecting the antihyperalgesic action of compounds that act on different targets**. Upon examination of the pathomechanism of thermal hyperalgesia we have concluded that the formation of **cyclooxygenase products and bradikinin as well as the TRPV1 receptor played important role in the development of heat threshold drop.**

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