INVESTIGATION OF THERMAL HYPERALGESIA AND ANTINOCICEPTIVE EFFECTS OF DRUGS WITH MEASUREMENT OF THE BEHAVIOURAL NOXIOUS HEAT THRESHOLD IN THE RAT

PhD thesis

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1. INTRODUCTION

1.1. General features and types of nociceptors

Nociceptors are specialized nerve endings detecting harmful stimuli and are peripheral terminals of a distinct – nociceptive – population of primary afferent neurons. Nociceptors can be classified according to their size and the existence or absence of the myelin sheet of their axons as myelinated Aδ nociceptors and unmyelinated, slowly-conducting C nociceptors (for review see Lynn, 1994; Woolf and Ma, 2007). Concerning activating stimuli there are mechano-, thermo- and chemonociceptors. Nociceptors responding to only one kind of these stimuli are called unimodal whereas those responding to more types are called polymodal nociceptors. Polymodal nociceptors play an outstanding role in detecting noxious heat stimuli and their membranes contain the transient receptor potential vanilloid type 1 (TRPV1) receptor which is the pharmacological receptor for capsaicin, the pungent compound of chilli pepper. Primary afferents can be divided pharmacologically into capsaicin-sensitive, polymodal and capsaicin-insensitive groups. A significant sub-group of the former neurons contains neuropeptides (for review see Holzer, 1991; Maggi, 1995; Szolcsányi, 1996; Szállási and Blumberg, 1999). These peptidergic nociceptors have dual function: the classical *afferent* function is activation by stimuli, depolarisation and the transmission of information to the central nervous system, whereas the neuropeptides released from nerve endings upon activation mediate local *efferent* effects. The efferent function is responsible for numerous reactions in neurogenic inflammation (Jancsó et al., 1967; 1968; Maggi, 1995; Szolcsányi, 1996).

1.2. Heat-sensitive ion channels

The TRPV1 receptor was the first putative (Szolcsányi and Jancsó-Gábor, 1975) and proved (Bevan and Szolcsányi, 1990; Szállási et al., 1993; 1995) capsaicin- and heat-sensitive ligand-gated ion channel permeable to Na^+, K^+ and Ca^{2+} ions that was cloned in 1997 (Caterina et al., 1997). This receptor is activated by heat stimuli (≥ 43 °C) and by several chemical agents as well (see lower). Among these resiniferatoxin (RTX) is also a pungent plant-derived irritant, an ultrapotent capsaicin analogue (Szolcsányi et al., 1990; Szállási et Blumberg, 1999). Moreover, several endogenous mediators (low pH, bradykinin, ATP, anandamide, lipoxygenase products etc.) proved to be able to activate the TRPV1 receptor. Surprisingly, TRPV1 receptor knock-out mice still detect noxious heat stimuli but

inflammatory thermal hyperalgesia is lacking in them (Caterina et al., 2000; Davis et al., 2000; Woodbury et al., 2004; Bölcskei et al., 2005; Zimmermann et al., 2005). A few TRPV1 receptor agonists (capsaicin, RTX) have a special ability to induce a sustained refractory state called sensory desensitization after the initial excitatory effect (Jancsó, 1959; Holzer, 1991; Szolcsányi, 1993; 2004; Szállási and Blumberg, 1999). Some agents are known as antagonists of the TRPV1 receptor. Among them capsazepine is a competitive antagonist of capsaicin and RTX, whereas ruthenium red blocks the ion channel part of the receptor. Iodo-resiniferatoxin (I-RTX) is a novel TRPV1 receptor antagonist that is more potent than capsazepine (Wahl et al., 2001; Rigoni et al., 2003).

Further five TRPV receptor types were identified after the discovery of TRPV1, so the TRPV receptor family consists of six members (TRPV1-6), of which TRPV2, TRPV3 and TRPV4 proved to be heat-sensitive with different activation thresholds (for review see Benham et al., 2003, Dhaka et al., 2006).

1.3. Features of thermonociception and thermal hyperalgesia

Strictly speaking neither pain nor effects of analgesics can be measured in animal tests because we do not know what the animals are actually sensing. We can only indirectly obtain information on the basis of the behaviour of the animal: if an animal shows an avoiding, averting or escaping response (nocifensive reaction) or vocalizes in response to a stimulus that is painful in humans, it is reasonable to assume that the animal feels pain. In these experimental conditions the term of nociception replaces semantically the term of pain. Based on the nature of the activating stimulus we can speak about mechano-, thermo- or chemonociception. The decrease of nociception by chemical or physical methods is called antinociception. Hyperalgesia (thermal, mechanical or chemical, according to the stimulus applied) refers to an increased response to a noxious stimulus. When the sensitivity of the organism for a stimulus is increased to such a level that a nocifensive response is evoked by a normally non-painful stimulus, the term allodynia is used. The effects decreasing hyperalgesia or allodynia are called antihyperalgesic or antiallodynic. These, together with the reduction of the response induced by noxious stimuli in non-sensitized state, are also included in the term of antinociceptive effect.

The relationship between the intensity of noxious heat stimulus and the magnitude of the response evoked is characterized by three parameters: (i) the heat threshold is the smallest stimulus intensity (lowest temperature) that can evoke a measurable response in the model

investigated; (ii) the maximal response is the level of the response the bigger of which cannot be evoked by further increase of the stimulus; (iii) the steepness of stimulus intensity–response relation characterizes the gain of encoding stimulus intensity. All the three parameters are characteristically changed in thermal hyperalgesia developing in pathological conditions: heat threshold is decreased; maximal response intensity and gain are increased.

1.4. Conventional methods for investigation of thermonociception

In traditional animal tests examining thermonociception such as the hot plate, tail-flick and paw-withdrawal (plantar) tests, latency of nocifensive reactions evoked by noxious heat stimuli of suprathreshold intensity is measured (for review see Le Bars et al., 2001). A constant temperature is set either on the hot plate or in water bath or a concentrated hot beam is directed onto the tail or paw of the animal and the time elapsed until the animal shows nocifensive behaviour (lifting, licking, shaking of the paw, jumping or withdrawal of the immersed tail or paw from water bath) is measured. One potential drawback of these methods is the alteration of latency upon repeated measurements. Latency may decrease or increase because of sensitizing effects of previously applied supratreshold stimuli or partial desensitizing effects of former stimuli. Therefore the reproducibility of the measurement of latency is limited, especially in tests using short periods of time (Gamble and Milne, 1989; Milne and Gamble, 1989; Carstens and Wilson, 1993; Plone et al., 1996; Sandkühler et al., 1996). The obvious disadvantage of these methods is that the measured latency cannot be compared with the results of single unit recording or patch-clamp experiments in which not latency but activation threshold is typically determined. In human studies noxious heat threshold is the typically measured parameter (Hardy et al., 1950; Szolcsányi, 1977; Meyer and Campbell, 1981; La Motte et al., 1982; Sycha et al., 2003).

It can be seen clearly that latency is a parameter that reflects the intensity of the response to a constant, suprathreshold heat stimulus. It would seem plausible to employ methods based on the other parameter of thermonociception, the heat threshold, especially because the measurement of the noxious threshold is a widely accepted method for the investigation of mechanonociception e.g. in the Randall–Selitto test. Furthermore, measurement of the heat threshold causes smaller degree of suffering for the animals than latency measurement does. Surprisingly, there are very few publications reporting on results obtained by measurement of heat threshold in animals. The first documented approach for studying the noxious heat threshold in animals was published by Szolcsányi who modified the

water bath paw-withdrawal test for threshold measurement (Szolcsányi, 1985; 1987). Hunskaar et al. (1986) modified the hot plate test by developing an increasing temperature hot plate which made possible the precise determination of the noxious heat threshold temperature. This method proved to be sensitive enough to reveal the thermal antinociceptive effect of morphine, paracetamol and acetylsalicylic acid (Hunskaar et al., 1986), but the effects of capsaicin or other TRPV1 receptor agonists have not been examined and no hyperalgesia test employing an increasing temperature hot plate has been elaborated. Practically, this method was forgotten.

On the basis of the above-mentioned facts it seemed reasonable to elaborate paradigms based on measurement of the noxious heat threshold and to investigate their pharmacological modulation.

2. AIMS

The aim of the present work was to investigate thermonociception by measurement of the behavioural noxious heat threshold. As no equipment suitable for measurement of the heat threshold was commercially available, a newly developed increasing temperature hot plate (ITHP) developed in an industrial cooperation was used. The aim of the first series of experiments was to biologically validate this new equipment. In frame of this, a new model of heat hyperalgesia based on decrease of the noxious heat threshold and suitable to assess thermal antihyperalgesic effects of drugs was elaborated. The aims were the following:

a. To assess the reproducibility of heat threshold by measuring the variability of heat threshold upon repeated measurements in untreated rats.
b. To assess the effects of reference analgesics on heat threshold and thereby determine the ability of this method to detect the direct thermal antinociceptive effect of drugs.
c. To work out a novel hyperalgesic test based on heat threshold drop by employing agents that are able to decrease the noxious heat threshold.
d. To investigate the thermal antihyperalgesic effects of reference analgesics by measuring the effect of classical analgesics on the threshold drop induced by TRPV1 receptor agonist.
e. To decide whether the sensory desensitizing effect of TRPV1 receptor agonists can be determined with the ITHP.

Thereafter, the effects of lipid compounds on noxious heat threshold were examined with the ITHP, too. The following fatty acid amides were analyzed: the recently identified TRPV1 receptor agonist *N*-oleoyldopamine (OLDA) and its two newly synthesized analogues, 3-methyl-*N*-oleoyldopamine (3-MOLDA) and 4-methyl-*N*-oleoyldopamine (4-MOLDA), as well as *N*-oleoylethanolamide (OEA). Further aim was to examine the effect of the dual cannabinoid/TRPV1 receptor agonist anandamide. Finally, we wanted to know whether the noxious heat threshold decreases in a subacute inflammatory state induced by carrageenan.

3. METHODS

3.1. Instruments

At an early stage of the experiments a novel computer-driven ITHP was used that had been developed in cooperation with the Supertech Ltd., Pécs. From 2003, a novel commercially available „incremental hot plate" instrument (IITC Inc. Life Science, Woodland Hills, CA, USA) was used which can work either in increasing temperature or constant temperature mode. This equipment was employed in our recent experiments (3rd own paper). The „cut-off" temperature was set to 50 °C with both instruments.

3.2. Animals

Most experiments were conducted on female Wistar rats weighing 140–200 g. In a limited number female C57BL6 mice (28–42 g) lacking the gene for the TRPV1 receptor (knock-out, $^{-/-}$, a generous gift from Dr. John B. Davis, GlaxoSmithKline, Harlow, UK) as well as their wild-type littermates ($^{+/+}$) were used. The animals were brought to the laboratory the day before the experiment and were provided with food and water *ad libitum*.

The experiments were performed according to the IASP (International Association for the Study of Pain) ethical guidelines (Zimmermann, 1983) and approved by the Ethical Committee of the University of Pécs.

3.3. Measurement of the noxious heat threshold

The experiments were conducted on female rats because the noncifensive response induced by heating the surface of the scrotum of male rats would have disturbed the

observations directed to the nocifensive response of the paw. The experiments were performed in a sound-attenuated and air-conditioned laboratory. All animals were tested only in one series of measurements. One heat threshold measurement (see below) was performed for adaptation purposes. Adaptation means that the animal was acclimatized to the method of the test with one measurement the result of which was not included in the analysis.

The rat was placed into the observation chamber on the plate that had a starting temperature of about 30 °C. Then the plate was heated up at a rate of 6 °C/min until the animal showed nocifensive behaviour involving either hind paw. The typical response was paw licking, while shaking and lifting of the paw or jumping was observed very rarely. Immediately after the occurrence of nocifensive behaviour heating was stopped and the animal was removed from the plate. The plate temperature evoking any of these nocifensive reactions confined to any paw was regarded as the noxious heat threshold of the animal. Following recording of the threshold temperature, the plate was cooled down to 30 °C by placing an ice-cold steel cover on it. The heat threshold measurement was repeated in 30 min and the mean of the two thresholds was considered as the control noxious heat threshold of the animal.

In those series of experiments in which a heat threshold-decreasing agent was administered by unilateral intraplantar injection, the control heat threshold of one of the hind paws was determined using a starting plate temperature of 15 °C and a heating rate of 12 °C/min (it is explained by the high degree and short duration of threshold drop; find further details in the Results). In the first series of measurements heating of the plate was continued until a nocifensive response occurred on either hind paw of the animal. In 30 min heating of the plate was maintained until the nocifensive behaviour occurred on the same paw, irrespectively whether the response of the other paw occurred or not. The mean of these two values was regarded as the control heat threshold of the hind paw.

3.4. Materials

RTX (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in ethanol and the stock solution (1 mg/ml) was sequentially diluted to 0.48 μM with physiological saline. I-RTX (Tocris Cookson Ltd, UK) was dissolved and diluted similarly to obtain a final solution of 10 or 1 μM. Morphine hydrochloride (Ph. Hg. VII) and diclofenac sodium (Research Biochemicals International, Natick, MA, USA) were dissolved in physiological saline. Paracetamol (Ph. Hg. VII) was dissolved in 12.5 % 1,2-propanediol (it was first dissolved in

one part of pure 1,2-propanediol by a short heating and then diluted with seven parts of physiological saline). α,β-methylene-ATP and PPADS (both from Sigma Chemical Co., St. Louis, MO, USA) were dissolved in physiological saline. OLDA (Tocris Cookson Ltd, UK) was dissolved in 10 % ethanol, 10 % TWEEN and 80 % physiological saline and the stock solution (10 mM) was diluted sequentially with saline. 3-MOLDA-t and 4-MOLDA were synthesized in the Chemical Research Centre of the Hungarian Academy of Sciences, Budapest. Stock solution of 3-MOLDA, 4-MOLDA and OEA (10 mM for each) were prepared with DMSO and further diluted in the appropriate buffers as required. OEA solutions were made fresh before every experiment. The systemic effect of agents and their solvents were examined after intraperitoneal (i.p.) injections (0.3 ml/100 g). Local effects were induced by intraplantar (i.pl.) injections (50 or 100 µl). The effects of agents were compared with their solvents. The observer was blind to the solution administered.

3.5. Statistical analysis

For comparison of data obtained in the same animals before and after drug administration, the Student's t-test for paired samples was employed. For comparison of the effects of the agents with those of their solvents, the Student's t-test for unpaired samples was used. For statistical analysis of repeated measurements analysis of variance (ANOVA) followed by Newman–Keuls *post hoc* test was used. Statistically significant differences were regarded in case of $P<0.05$.

4. RESULTS

4.1. Measurement of the noxious heat threshold and its pharmacological modulation by analgesics (1^{st} own paper)

4.1.1. Reproducibility of the measurement of noxious heat threshold

As the first step of the validation of the ITHP, the noxious heat threshold of untreated rats was determined and found to be 45.3±0.3 °C (n=36). In order to assess the within-group variability of heat threshold, repeated measurements were made in three groups of untreated animals at intervals of 5 min, 30 min or 24 h. Highly reproducible threshold values without significant alterations were obtained. The lack of statistical significance refers to the difference between (i) thresholds of the same paw measured at various time points; (ii) thresholds of the animal (defined as the lower value of the two paw thresholds) measured at

various time points; (iii) threshold of the left and right paws measured at a given time point. Generally, the comparison of the control heat threshold in various groups of animals revealed no significant differences indicating that the inter-group variability of the threshold was also negligible.

4.1.2. Effect of morphine, diclofenac and paracetamol on the noxious heat threshold

In order to measure the effect of morphine, diclofenac and paracetamol on the noxious heat threshold, the drug or its vehicle was administered i.p. (0.3 ml per 100 g). Threshold measurement was repeated 30 min later.

Each of these analgesics caused an elevation of the noxious heat threshold of rats in a dose-dependent manner, whereas their solvents failed to cause a significant effect. The minimum effective doses of morphine, diclofenac and paracetamol causing a significant increase of the heat threshold were 3, 10 and 200 mg/kg, respectively.

4.2. Effect of resiniferatoxin (RTX) on the heat threshold and its pharmacological modulation (1^{st} own paper)

4.2.1. Thermal hyperalgesia model based on heat threshold drop induced by activation of the TRPV1 receptor

The effect of unilateral i.pl. injection of RTX (0.048 nmol, 100 µl) on the heat threshold in the rat was tested. In one series of experiments (n=12) the thresholds of both the treated and untreated hind paw were measured 5, 10, 15, 20 and 25 minutes after the RTX administration. The solvent of RTX induced no nocifensive response and failed to alter the heat threshold.

After an i.pl. injection of RTX, an acute nocifensive response (licking and shaking of the treated paw) was observed. These reactions usually lasted for less than 5 min. When testing the RTX-treated animals on the ITHP, lifting of the treated hind paw was the first heat-evoked behavioural reaction on almost every occasion which was rarely seen under control conditions. Paw lifting appeared at low plate temperatures in the innocuous range of 34–41 °C and was followed by the paw licking reaction at higher temperatures. The thresholds for both reactions reached their minima at the 5 min measurement with gradual recovery afterwards. By 25 min after RTX injection, the threshold returned to the control value.

4.2.2. Effect of morphine, diclofenac and paracetamol on the RTX-induced threshold drop

In order to measure the effect of morphine, diclofenac and paracetamol on the RTX-induced threshold drop an actual vehicle control was used, as one half of the group of animals received the analgesic i.p. (0.3 ml per 100 g) and the other half was treated with its solvent 25 min prior to RTX (0.048 nmol per paw) administration. The heat threshold of only the treated hind paw was determined 5, 10, 15 and 20 min after RTX injection. The dose–response relationship for the inhibitory effect of drugs on the RTX-induced drop of heat threshold was determined and percentage inhibition of threshold drop was calculated either on the basis of threshold drop 5 min after RTX injection or with the sum of threshold drops at 5, 10, 15 and 20 min.

Morphine, diclofenac and paracetamol exerted a dose-dependent inhibitory effect on the RTX-induced threshold drop with a minimal effective dose of 1, 1 and 100 mg/kg, respectively.

4.2.3. Investigation of the TRPV1 receptor antagonist iodo-resiniferatoxin (I-RTX) with the ITHP

I-RTX is a recently developed TRPV1 receptor antagonist that proved to be more potent than capsazepine (Wahl et al., 2001). Until the time of our experiments, its TRPV1 receptor antagonistic effect under *in vivo* conditions had been investigated only to a very limited extent (Wahl et al., 2001; Undem and Kollarik, 2002).

In the first series of experiments the acute and long-term effect of I-RTX on the noxious heat threshold were tested. Briefly, I-RTX was administered i.pl. (0.1 or 1 nmol per paw in 100 μl) into both hind paws. Bilateral treatment was needed because the possible heat threshold elevation produced by I-RTX could have been disturbed by the nocifensive reaction of the untreated paw. Heat threshold determinations were performed 5, 10, 15, 20, 30, 40 and 50 min after I-RTX injection as well as at 1, 2, 4 and 6 h during the day and subsequently daily for 5 days. Following I-RTX treatment no nocifensive behaviour was observed and no alteration of the heat threshold was measured either acutely or on the long term.

In another series of experiments, the possible inhibitory effect of I-RTX on the heat threshold drop induced by RTX and its selectivity for TRPV1 receptor were tested. Concerning the latter the effect of I-RTX on the threshold drop caused by α,β-methylene-ATP, a drug that does not act on TRPV1 receptor, was studied. I-RTX (0.05 nmol per paw in

50 ml) or its solvent was given i.pl. 5 min prior to either RTX (0.048 nmol per paw) or α,β-methylene-ATP (0.3 μmol per paw) injection to the same paw. Heat threshold determinations of the treated paws were performed 5, 10, 15 and 20 min after the administration of RTX and α,β-methylene-ATP.

I.pl. pre-treatment with I-RTX (0.05 nmol, 5 min prior to RTX) diminished the heat threshold drop induced by RTX, the percentage inhibition values being 51 % (according to threshold drop at 5 min) and 64 % (on the basis of sum of threshold drops). I.pl. injection of α,β-meATP (0.3 μmol) also decreased the heat threshold of the treated paw to 39.5±1.2 °C at 5 min and 42.0±0.9 °C at 10 min. At later time points no significant threshold alteration was measured. The effect of α,β-methylene-ATP was measured also following a 5 min i.pl. pre-treatment with the P2 purinergic receptor antagonist pyridoxalphosphate-6-azophenyl-2'4'-disulphonic acid (PPADS, 0.15 μmol per paw in 50 μl) or its solvent. PPADS reduced the threshold-lowering effect of α,β-methylene-ATP, the percentage inhibition being 83 % at 5 min ($n=8$, $P<0.01$). I-RTX pre-treatment (0.05 nmol per paw, 5 min before) failed to inhibit the α,β-methylene-ATP-induced drop of the heat threshold.

4.2.4. Long-term effect of RTX on the noxious heat threshold

As a part of validation of the ITHP the long-term effect of RTX on the noxious heat threshold was studied by administration of RTX (0.048 nmol/100 ml per paw) or its solvent (0.03 % ethanol) bilaterally to eliminate the disturbing effect of the reaction of the untreated paw. Heat threshold determinations were performed 5, 10, 15, 20 and 25 min after RTX injection as well as at 1, 2, 4 and 6 h and subsequently daily for a week. Threshold was determined according to the first-reacting hind paw. If the cut-off temperature was reached, the animal was immediately removed from the plate and the value of 50 °C was used in the analysis.

The heat threshold-elevating effect of RTX was manifest as early as 1 h after administration and lasted for several days (maximum increase approx. 3.0 °C). By day 6 the heat threshold returned into the control range. The solvent of RTX failed to exert any effect on the heat threshold.

4.2.5. Comparison of noxious heat threshold in TRPV1 receptor knock-out and wild-type mice

In this series of experiment the noxious heat threshold of TRPV1 knock-out mice and their wild-type littermates was compared. For this reason female 28–42 g wild-type ($^{+}/^{+}$) C57BL6 mice and their TRPV1 knock-out ($^{-}/^{-}$) pairs were tested. Heat threshold measurements were performed in the same way as in the rats and licking or shaking of either hind paw was regarded as the end point of the measurement.

No significant difference was found between the noxious heat thresholds of the two animal populations (wild-type 45.2±0.4 °C and TRPV1 knock-out 45.6±0.5 °C).

4.3. Investigation of the effect of lipid compounds on the noxious heat threshold

4.3.1. Effect of *N*-oleoyldopamine (OLDA) on the noxious heat threshold (2^{nd} own paper)

Several agents are known to be able to activate the TRPV1 receptor (for review see Pingle et al., 2007), the endogenous ligand of TRPV1, however, remained enigmatic. Anandamide and 12-(*S*)-hydroperoxy-eicosatetraenoic acid (12-HPETE) have been proposed to serve as endogenous ligands for the TRPV1 receptor (Zygmunt et al., 1999; Hwang et al., 2000). Recently, a novel endogenous lipid, *N*-oleoyldopamine (OLDA), stucturally related to capsaicin, was reported to evoke nocifensive response after i.pl. administration that was antagonized by I-RTX showing the mediator role of TRPV1 receptor (Chu et al., 2003).

In our experiments the effect of OLDA on the noxious heat threshold and the receptorial background of the behavioural response to OLDA were studied. OLDA (5 nmol) was administered i.pl. after i.pl. pre-treatment with I-RTX (0.05 nmol) or its solvent in rats. OLDA immediately induced an overt nociceptive behaviour which disappeared within 10 min. After cessation of this reaction OLDA evoked a drop of the heat threshold by 6–9 °C. By 60 min after OLDA injection the heat threshold returned to control. Local I-RTX pre-treatment significantly diminished the noxious heat threshold-lowering effect of OLDA.

Thereafter the nocifensive effect of OLDA (50 nmol/50 µl i.pl.) was investigated in TRPV1 knock-out mice compared with wild-type littermates. The duration of hind paw lifting and licking was measured. In mice, similarly to rats, OLDA evoked an immediate and overt nocifensive reaction, its duration being significantly shorter in TRPV1 receptor knock-out mice compared to their wild-type controls. However, in TRPV1 receptor knock-out mice

OLDA was still able to elicit some nocifensive response unlike its solvent that was without effect.

4.3.2. Actions of 3-methyl-*N*-oleoyldopamine (3-MOLDA), 4-methyl-*N*-oleoyldopamine (4-MOLDA) and *N*-oleoylethanolamide (OEA) on the rat TRPV1 receptor (3rd own paper)

To continue our experiments with OLDA that proved to be a TRPV1 receptor agonist, the effects of two newly synthesized methylated derivatives of OLDA, 3-methyl-*N*-oleoyldopamine (3-MOLDA) and 4-methyl-*N*-oleoyldopamine (4-MOLDA) on TRPV1 receptor were investigated, first of all with the measurement of noxious heat threshold. In the light of the contradictory data obtained with a related fatty acid amide *N*-oleoyl-ethanolamide (OEA) (Ahern, 2003; Wang et al., 2005; LoVerme et al., 2006; Suardíaz et al., 2007) its effects on the TRPV1 receptor were also investigated.

In this series of experiments the possible TRPV1 receptor agonistic effect of the unilateral i.pl. injection of 3-MOLDA, 4-MOLDA or OEA (100 µl) was examined after control heat threshold measurement (incremental hot plate, IITC Inc. Life Science, USA). For revealing an antagonistic effect of compounds at the TRPV1 receptor, the previously mentioned RTX heat allodynia/hyperalgesia test was used.

3-MOLDA (5 nmol i.pl.) evoked an instantaneous nocifensive reaction which disappeared within 10 min. The subsequent threshold measurements revealed that this dose of 3-MOLDA significantly decreased the heat threshold with a maximum drop of threshold observed 15 min after administration. The heat threshold-lowering effect of 3-MOLDA was inhibited by pre-treatment with the TRPV1 receptor antagonist I-RTX (0.05 nmol i.pl., 5 min before). In contrast to 3-MOLDA, 4-MOLDA and OEA failed to evoke nocifensive behaviour or alter the heat threshold up to i.pl. applied doses of 5 nmol, respectively.

Since neither 4-MOLDA nor OEA evoked nocifension or a measurable drop of heat threshold, they were tested for possible TRPV1 receptor antagonistic action against RTX, the reference TRPV1 receptor agonist in this assay. Pre-treatment with the middle and highest dose (1.5 and 5 nmol) of 4-MOLDA (i.pl., 5 min before) diminished the heat threshold-lowering effect of RTX (0.05 nmol i.pl.). OEA pre-treatment (5 min before, 0.5, 1.5 and 5 nmol) also reduced the RTX-induced heat threshold drop in a dose-dependent manner with percentage inhibition values (calculated with the sum of threshold drops) ranging from 2.6 to 84.2 % and with an ID_{50} value of 1.4 nmol.

4.3.3. The effect of anandamide on the noxious heat threshold (3rd own paper)

A further fatty acid amide, arachidonyl-ethanolamide also known as anandamide is the endogenous ligand for cannabinoid receptors, especially for CB_1 receptor (for review see Pertwee, 2001). Anandamide proved to have antinociceptive effects in several *in vivo* animal models because it decreased inflammatory thermal and mechanical hyperalgesia via CB_1 receptor activation (Calignano et al., 1998; Jaggar et al., 1998; Richardson et al., 1998; Farquhar-Smith et al., 2002). According to recent data anandamide can also activate TRPV1 receptors *in vitro* (Zygmunt et al., 1999; Smart et al., 2000), however at much higher concentrations (Németh et al., 2003; Ahluwalia et al., 2003). Based on these facts anandamide is considered as a dual, cannabinoid/TRPV1 receptor agonist. The aim of this series of the assay was to examine the effect of anandamide on TRPV1 receptor in our *in vivo* model.

Anandamide (0.03–0.3 nmol, i.pl.) failed to evoke nocifensive behaviour or alteration of the unconditioned heat threshold measured with the incremental hot plate. Thereafter the effect of anandamide pre-treatment (5 min before) was studied on the heat threshold drop induced by RTX. Anandamide (0.03 nmol/50 µl) diminished the heat threshold-lowering effect of RTX (0.05 nmol i.pl.). The inhibitory effect of anandamide was abolished by co-administration of the CB_1 cannabinoid receptor antagonist SR141716A (0.18 nmol i.pl).

4.4. Effect of subacute inflammation induced by carrageenan on the noxious heat threshold

Our previous experiments have shown that several agents can decrease the heat threshold in rats. RTX, OLDA, 3-MOLDA and α,β-methylene-ATP act on receptors (TRPV1 or P2X3) the activation of which results in an inflammatory response. The thermal hyperalgesia induced by these agents includes a decrease in both latency and heat threshold. The question arises: do these two parameters of thermal hyperalgesia always alter in a parallel manner? For this purpose a subacute inflammatory model was chosen in which inflammation lasting for several hours was induced by i.pl. injection of carrageenan. Unequivocal evidence shows that this agent evokes a decrease in latency of paw withdrawal in conventional thermonociceptive tests (Hargreaves et al., 1988). To make use of the advantage of the incremental hot plate (IITC Inc. Life Science) that heat threshold can be measured in increasing temperature mode and latency can be measured in constant temperature mode, the effect of carrageenan both on the noxious heat threshold and on the latency of nocifensive

response evoked by a constant suprathreshold heat stimulus were measured in the same population of rats.

Before administration of carrageenan heat threshold and latency of paw licking at constant plate temperature of 50 °C of one of the hind paws in the same animals were determined. Thereafter carrageenan (3 %, 100 μl) was applied into the paw for which the control values had been determined previously. The carrageenan-induced inflammatory reaction of the hind paw developed within an hour and lasted for 3–4 hours. Surprisingly, carrageenan failed to decrease the noxious heat threshold whereas in the same animals it was able to decrease the latency of paw licking induced by suprathreshold (50 °C) heat stimulation.

5. DISCUSSION

The newly developed and validated ITHP equipment used in the experiments proved to be a reliable tool for measurement of the noxious heat threshold and its modulation by drugs in conscious unrestrained rats. An important feature of the ITHP method is the excellent reproducibility. A further advantage of this method over the conventional hot plate test is that it measures the real noxious heat threshold temperature that can be compared to other heat thresholds measured in electrophysiological and human psychophysical tests.

The present results demonstrate that in addition to morphine, the nonselective cyclooxygenase inhibitor diclofenac and the nonopioid analgesic paracetamol are also capable of elevating the noxious heat threshold in a dose-dependent manner, whereas the classical constant temperature hot plate test can only reveal the antinociceptive effect of opioid analgesics being largely insensitive to cyclooxygenase inhibitors (for details see Vogel and Vogel, 1997; Le Bars et al., 2001).

The present study determined for the first time the real heat threshold-lowering action of a TRPV1 receptor agonist in conscious unrestrained animals. Intraplantar injection of RTX evoked a profound drop of the heat threshold and this response turned out to be a useful model for testing the effects of analgesic drugs. The minimal effective doses of morphine, diclofenac and paracetamol that inhibited RTX evoked hyperalgesia are proved to be smaller than the minimal effective doses of these drugs causing elevation of the unconditioned heat threshold. We can conclude that the measurement of heat threshold drop induced by RTX with the ITHP is a novel hyperalgesia model that is very sensitive to analgesics.

The long-term but still reversible sensory desensitizing effect of RTX could also be demonstrated using the ITHP as a long-lasting increase of heat threshold.

I-RTX was found to be a potent and selective TRPV1 receptor antagonist *in vivo* upon i.pl. administration. I-RTX decreased the heat threshold drop induced by RTX but did not alter the threshold drop induced by α,β-methylene-ATP. The lack of a long-term heat threshold-elevating effect of I-RTX indicates that no significant metabolic conversion of I-RTX to RTX detectable with this behavioural read-out occurs. The α,β-methylene-ATP-induced threshold drop was inhibited by the P2 purinergic receptor antagonist PPADS indicating that P2 receptor activation was involved.

The heat threshold of TRPV1 receptor knock-out mice was found not different from that of their wild-type counterparts and I-RTX failed to elevate the heat threshold in rats indicating that the TRPV1 receptors are unlikely to be involved in determination of the behavioural noxious heat threshold under physiological conditions.

OLDA behaving as a TRPV1 receptor agonist decreased the heat threshold and induced a nocifensive response. A part of this latter pro-nociceptive effect is due to activation of TRPV1 receptors, but some other, non-identified mechanism(s) may also operate.

The present assay has shown that 3-MOLDA behaved as a TRPV1 receptor agonist while 4-MOLDA and OEA appeared to be antagonists (or very weak partial agonists) of the TRPV1 receptor. It is worth emphasizing that 3-MOLDA and 4-MOLDA, two methylated derivatives of the TRPV1 receptor agonist OLDA differing only in the position of the methyl group on the dihydroxylated aromatic ring, behaved as a TRPV1 receptor agonist and antagonist, respectively. These results show that even a slight chemical difference can have a dramatic influence on the effect of the fatty acid amide compounds on the TRPV1 receptor protein.

The other fatty acid amide investigated, anandamide, also inhibited the heat threshold-lowering effect of RTX, i.e. it exerted a thermal antihyperalgesic action. Anandamide is the endogenous ligand of cannabinoid receptors with higher affinity for the CB_1 receptors (for a review see Pertwee, 2001). In addition, anandamide is capable of activating the TRPV1 receptor (Zygmunt et al., 1999; Smart et al., 2000), albeit at much higher concentrations than those required for stimulation of the cannabinoid receptors (Németh et al., 2003; Ahluwalia et al., 2003). Our results do not support the hypothesis that anandamide can function as an endogenous, physiological activator of TRPV1 receptors because it inhibited the effect of the TRPV1 receptor agonist RTX and this inhibitory effect was abolished by a CB_1 receptor

antagonist, showing that the inhibitory effect of ANA is mediated by CB_1 cannabinoid receptors.

The ITHP method failed to demonstrate a drop of the noxious heat threshold in subacute inflammation induced by carrageenan. However, carrageenan decreased the latency of nocifensive response of the same animals induced by suprathreshold heat stimulation using the same instrument in the constant temperature mode. We can conclude that the two components of inflammatory thermal hyperalgesia – the decrease of latency and the drop of heat threshold – are not necessarily altered in a parallel manner.

Last but not least, it should be emphasized that although measurement of the noxious heat threshold proved to be a suitable method for the investigation of both theoretical and practical problems, further examinations are necessary to learn the advantages and disadvantages of this approach. Employing both classical tests based on measurement of latency and new assays based on assessing the noxious heat threshold will be needed for development of long awaited new types of analgesics.

6. SUMMARY OF THE NEW FINDINGS PRESENTED IN THE THESIS

1. The behavioural noxious heat threshold can be measured with the increasing temperature hot plate reliably and reproducibly.

2. The TRPV1 receptor is not involved in determination of the behavioural noxious heat threshold under physiological conditions.

3. The direct thermal antinociceptive effects of morphine, diclofenac and paracetamol can be demonstrated by measuring the elevation of the (unconditioned) noxious heat threshold.

4. Measurement of the noxious heat threshold is suitable for revealing the acute, thermal hyperalgesic (RTX, OLDA, 3-MOLDA) as well as the sustained, thermal antinociceptive action (based on sensory desensitization) of TRPV1 receptor agonists (RTX).

5. The heat hyperalgesia/allodynia model based on the drop of noxious heat threshold induced by i.pl. RTX is equally suitable for investigation of standard analgesics (morphine, diclofenac and paracetamol), TRPV1 receptor antagonists (I-RTX, 4-MOLDA, OEA) and CB_1 cannabinoid receptor agonist (anandamide).

6. With investigation of the subacute inflammatory condition induced by carrageenan it has been demonstrated that the two components of inflammatory thermal hyperalgesia – the decrease of latency and the drop of heat threshold – are not necessarily coupled to each other which reflects their different pathophysiological regulation.

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8.1. Full-length articles

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