British Journal of Pharmacology (2003) 139, 49–58 © 2003 Nature Publishing Group All rights reserved 0007–1188/03 $25.00
www.nature.com/bjp

Effect of resiniferatoxin on the noxious heat threshold temperature in the rat: a novel heat allodynia model sensitive to analgesics

[1]Róbert Almási, [1]Gábor Pethö, [1]Kata Bölcskei & *,[1]János Szolcsányi

[1]Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, Neuropharmacology Research Group of the Hungarian Academy of Sciences, University of Pécs, Szigeti u. 12, H-7624 Pécs, Hungary

1 An increasing-temperature hot plate (ITHP) was introduced to measure the noxious heat threshold (45.3 ± 0.3°C) of unrestrained rats, which was reproducible upon repeated determinations at intervals of 5 or 30 min or 1 day.

2 Morphine, diclofenac and paracetamol caused an elevation of the noxious heat threshold following i.p. pretreatment, the minimum effective doses being 3, 10 and 200 mg kg^{-1}, respectively.

3 Unilateral intraplantar injection of the VR1 receptor agonist resiniferatoxin (RTX, 0.048 nmol) induced a profound drop of heat threshold to the innocuous range with a maximal effect (8 – 10°C drop) 5 min after RTX administration. This heat allodynia was inhibited by pretreatment with morphine, diclofenac and paracetamol, the minimum effective doses being 1, 1 and 100 mg kg^{-1} i.p., respectively.

4 The long-term sensory desensitizing effect of RTX was examined by bilateral intraplantar injection (0.048 nmol per paw) which produced, after an initial threshold drop, an elevation (up to 2.9 ± 0.5°C) of heat threshold lasting for 5 days.

5 The VR1 receptor antagonist iodo-resiniferatoxin (I-RTX, 0.05 nmol intraplantarly) inhibited by 51% the heat threshold-lowering effect of intraplantar RTX but not α,β-methylene-ATP (0.3 μmol per paw). I-RTX (0.1 or 1 nmol per paw) failed to alter the heat threshold either acutely (5 – 60 min) or on the long-term (5 days). The heat threshold of VR1 receptor knockout mice was not different from that of wild-type animals (45.6 ± 0.5 *vs* 45.2 ± 0.4°C).

6 In conclusion, the RTX-induced drop of heat threshold measured by the ITHP is a novel heat allodynia model exhibiting a high sensitivity to analgesics.
British Journal of Pharmacology (2003) **139,** 49 – 58. doi:10.1038/sj.bjp.0705234

Keywords: Increasing-temperature hot plate; noxious heat threshold temperature; resiniferatoxin; heat allodynia; morphine; diclofenac; paracetamol; sensory desensitization; iodo-resiniferatoxin; α,β-methylene-ATP

Abbreviations: I-RTX, iodo-resiniferatoxin; ITHP, increasing-temperature hot plate; α,β-meATP, α,β-methylene adenosine 5′-triphosphate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; RTX, resiniferatoxin; VR1, vanilloid receptor type 1

Introduction

In 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. These methods are suitable for measuring the antinociceptive effects of opioid analgesics but are largely insensitive to cyclooxygenase inhibitors and paracetamol (see Vogel & Vogel, 1997; Le Bars *et al*., 2001). In addition, reflex latency measured with these methods is difficult to compare with any of the parameters determined routinely in single-unit recording or patch-clamp experiments examining noxious heat responsiveness of nociceptive primary afferent neurons. While measurement of the nociceptive threshold is a widely accepted method for the investigation of mechanonociception (e.g. in the Randall – Selitto test), this approach has been exploited for studying thermonociception only in a very limited manner (see below). Examining the nocifensive behaviour of capsaicin-pretreated rats with the conventional hot plate and tail-flick tests led to controversial results (Szolcsányi, 1976; Obál *et al*., 1979; Hayes & Tyers, 1980; Gamse, 1982; Bittner & Lahann, 1984), although this agent desensitized to heat a major subgroup of primary afferent neurons, the polymodal nociceptors (Szolcsányi, 1987a, b) expressing the capsaicin VR1 receptor, which is also activated by noxious heat (Caterina *et al*., 1997; Tominaga *et al*., 1998). On the other hand, modifying the paw-withdrawal test for an approximate measurement of the noxious heat threshold temperature (by immersing the rat hindpaw in a water bath whose temperature was increased in a stepwise manner in 1°C units until paw withdrawal occurred) instead of the reflex latency revealed a clearcut dose-dependent thermal antinociceptive effect of capsaicin (Szolcsányi, 1985; 1987b). Hunskaar *et al*. (1986) modified the hot plate test by developing an increasing-temperature hot plate (ITHP), which made possible the 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

*Author for correspondence; E-mail: janos.szolcsanyi@aok.pte.hu

the effect of capsaicin or other VR1 receptor agonists has not been examined and this test has not become a standard method for investigation of the thermoanalgesic effect of drugs (Le Bars *et al*., 2001). Furthermore, according to our knowledge, no hyperalgesic test employing an ITHP has been elaborated.

Resiniferatoxin (RTX) is a naturally occurring phorbol compound acting as a highly potent agonist at the capsaicin VR1 receptor (see Szállási & Blumberg, 1999). Similar to capsaicin, RTX first selectively excites the VR1 receptor-expressing polymodal nociceptors, which is followed by a lasting impairment of the function of these neural elements often referred to as sensory desensitization (Szolcsányi, 1990; Szállási & Blumberg, 1999). Iodo-resiniferatoxin (I-RTX) is a recently developed VR1 receptor antagonist that proved to be more potent than capsazepine *in vitro*, but its VR1 receptor antagonistic effect under *in vivo* conditions has been investigated only to a very limited extent (Wahl *et al*., 2001; Undem & Kollarik, 2002). It is worth mentioning that the possibility that I-RTX is converted to RTX by deiodination under *in vivo* conditions cannot be ruled out and has not been investigated so far. Such a conversion could result in mixing of the VR1 receptor antagonistic property of I-RTX with VR1 agonism including sensory desensitization. Since the latter effect may manifest itself similarly to VR1 receptor antagonism, deiodination could render I-RTX less suitable for studying the role of the VR1 receptor.

The aim of the present study was first to validate our newly developed ITHP equipment and thereafter to work out a hyperalgesic test based on the decrease of the noxious heat threshold. For this latter purpose, RTX seemed to be an ideal candidate since the acute heat-sensitizing effect of VR1 receptor agonism is well known (Szolcsányi, 1977; Simone *et al*., 1987; Gilchrist *et al*., 1996; Yeomans *et al*., 1996). Furthermore, the long-term sensory desensitizing effect of RTX on the noxious heat threshold was also examined in order to determine whether the ITHP is suitable to reveal the lasting thermal antinociceptive effect of VR1 receptor agonism for which ambiguous results have been provided previously (Szolcsányi, 1976; Obál *et al*., 1979; Hayes & Tyers, 1980; Gamse, 1982; Bittner & Lahann, 1984). I-RTX's *in vivo* VR1 receptor antagonistic action (with special reference to its selectivity) and its own effect on the noxious heat threshold (both acutely and in the long term) were also investigated to detect any possible VR1 receptor agonistic action and/or conversion to RTX. The role of the VR1 receptor in determination of the behavioural noxious heat threshold of peripheral nociceptors was also investigated in VR1 receptor knockout mice using the ITHP.

A brief account of the present work appeared in the abstract form (Szolcsányi *et al*., 2001).

Methods

A novel computer-driven ITHP was used that had been developed in cooperation with the Supertech Ltd, Pécs, Hungary. In contrast to the classical constant-temperature hot plate, with which latency of nocifensive behaviour is measured, this equipment is suitable for the determination of the noxious heat threshold, that is, the lowest temperature evoking such behaviour. The equipment consists of three components: a metallic plate (20×15 cm) with the heating system underneath and a Plexiglas observation chamber above, a heat controlling unit and a PC. The heating system is controlled by a newly developed software that allows starting and stopping of the heating process as well as setting various heating rates. During the heating, the rise of the plate temperature was shown to be near-linear and the temperature difference between various points of the plate did not reach 0.5°C as measured by thermocouples. The cutoff temperature was set to 50°C.

The experiments were performed according to the international ethical guidelines (Zimmermann, 1983) and approved by the Ethical Committee of the University of Pécs. Experiments were conducted on female Wistar rats (Charles River Hungary Ltd, Budapest, Hungary) weighing 140 – 200 g and on female C57BI6 mice (28 – 42 g) lacking the gene for VR1 receptor (−/−, a generous gift from Dr John B. Davis, GlaxoSmith Kline, Harlow, U.K.) as well as their wild-type counterparts (+/+, Charles River Hungary Ltd, Budapest, Hungary) in a sound-attenuated and air-conditioned laboratory. All animals were tested only in one series of measurements.

Measurement of the control noxious heat threshold temperature and its modulation by analgesics

The animals were brought to the laboratory the day before the experiment and were provided with food and water *ad libitum*. One heat threshold measurement (see below) was performed for adaptation purposes the result of which was not used 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^{-1} until the animal showed nocifensive behaviour involving either hindpaw. The typical response was hindpaw licking, while shaking and lifting of the paw or jumping was observed very rarely. 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 animal was immediately removed from the plate, which was cooled down again 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 a limited number of experiments investigating the reproducibility of the heat threshold upon repeated measurements, the thresholds of both hindpaws were determined; that is, heating was continued after nocifensive reaction of either hindpaw occurred until the other one showed reaction as well.

The noxious heat threshold was determined also in mice lacking the gene for the VR1 receptor (−/−) as well as in their wild-type counterparts (+/+). Threshold determination was performed essentially in the same way as in the case of rats, the typical end point being licking or shaking of either hindpaw.

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). The observer was blind to the solution administered. Threshold measurement was repeated 30 min later using a starting temperature of 30°C and a heating rate of 6°C min^{-1}.

Investigation of the acute and long-term effect of RTX on the heat threshold and measurement of the effect of analgesics on the RTX-induced acute threshold drop

Prior to intraplantar RTX administration, the control heat threshold of one of the hindpaws was determined using a starting plate temperature of 15°C and a heating rate of 12°C min^{-1}. The reason for these changes was the massive heat threshold drop induced by RTX (see Results). During the first measurement, the heating of the plate was continued until a nocifensive reaction of either hindpaw occurred; however, upon the second determination 30 min later, heating was continued until a reaction of the hindpaw whose threshold was recorded in the first measurement was observed, regardless of the reactions of the other paw. The mean of the two values served as the control heat threshold of the examined paw. RTX (0.048 nmol (0.03 μg) per paw in 100 μl) or its solvent was injected under the plantar skin of the paw. In this series of experiments ($n = 12$), the heat thresholds of both the treated and untreated hindpaws were determined 5, 10, 15, 20 and 25 min after RTX injection.

Upon investigation of 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. This design ascertained that the variability of the magnitude of RTX-induced threshold drop in the various animal populations used on different occasions had biased the investigation to the minimum possible extent. The observer was blind to the solution administered. The heat threshold of only the treated hindpaw was determined 5, 10, 15 and 20 min after RTX injection.

In another series of measurements, the long-term effect of intraplantar RTX injection was studied by a bilateral administration of RTX (0.048 nmol per paw) or its solvent. 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. Threshold was determined according to the first-reacting hindpaw. If the cutoff temperature was reached, the animal was immediately removed from the plate and the value of 50°C was used in the analysis.

Investigation of the effect of I-RTX on the noxious heat threshold of control and sensitized rats

The experiments exploring the acute and long-term effect of I-RTX on the noxious heat threshold were conducted similarly as in the case of RTX. Briefly, I-RTX was administered intraplantarly (0.1 or 1 nmol per paw in 100 μl) into both hindpaws. The starting temperature was 15°C and the heating rate was 12°C min^{-1}. 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 and subsequently daily. Threshold was determined according to the first-reacting hindpaw.

In another series of experiments, I-RTX (0.05 nmol per paw in 50 μl) or its solvent was given intraplantarly 5 min prior to either RTX (0.048 nmol per paw) or α,β-methylene-ATP (α,β-meATP, 0.3 μmol per paw in 100 μl) injection to the same paw. The effect of α,β-meATP was measured also following a 5 min intraplantar pretreatment with pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 0.15 μmol per paw in 50 μl) or its solvent. The heat threshold of only the treated hindpaw was determined 5, 10, 15 and 20 min after RTX and at 5, 10 and 15 min following α,β-meATP administration.

Statistical analysis

For analysis of multiple measurements (threshold determinations at various intervals without drug treatment or at different time points during the acute or long-term effect of RTX, I-RTX or α,β-meATP) analysis of variance (ANOVA) followed by Newman – Keuls test was used. For comparison of two data groups, the Student's *t*-test for paired (heat threshold before *versus* after drug treatment) or unpaired samples (RTX- or α,β-meATP-induced threshold drop in the drug- *versus* solvent-pretreated animals; heat threshold in VR1 receptor knockout *versus* wild-type mice) was employed. In cases when the cutoff temperature was reached, the value of 50°C was used in the analysis.

Drugs and solutions

RTX (Sigma Chemical Co., St Louis, MO, U.S.A.) was dissolved in ethanol and the stock solution (1 mg ml^{-1}) was sequentially diluted to 0.3 μg ml^{-1} (0.48 μM) with physiological saline. I-RTX (Tocris Cookson Ltd, U.K.) was dissolved and diluted similarly to obtain a final solution of 10 or 1 μM. Morphine hydrochloride (pharmaceutical grade, Ph. Hg. VII) and diclofenac sodium (Research Biochemicals International, Natick, MA, U.S.A.) were dissolved in physiological saline. Paracetamol (pharmaceutical grade, 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-meATP and PPADS (both from Sigma Chemical Co., St Louis, MO, U.S.A.) were dissolved in physiological saline.

Results

Reproducibility of the measurement of noxious heat threshold in rats

The noxious heat threshold temperature of untreated rats was 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 (Figure 1). 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 and (iii) threshold of the left and right paws measured at a given time point. 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.

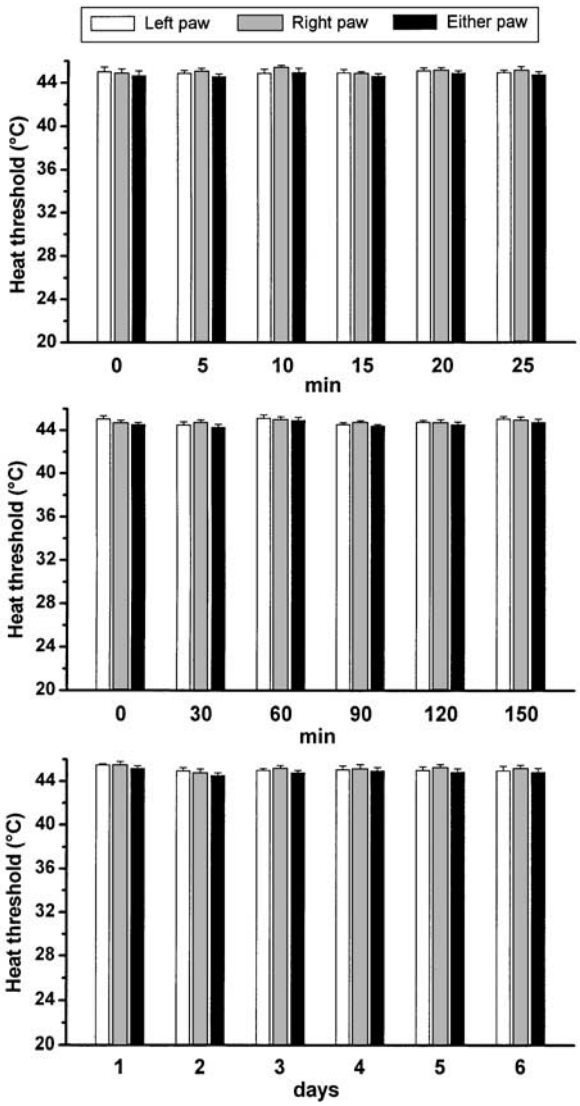

Figure 1 Reproducibility of the noxious heat threshold temperature upon repeated measurements in three different groups of untreated rats. Heat threshold of either paw (black columns) refers to the lower value of the left and right paws threshold, which represents the heat threshold of the animal. The heating rate was 12°C min^{-1} in the first group (upper panel) and 6°C min^{-1} in the second and third groups. Data are means with s.e.m. of 12 animals in each group. Statistical analysis revealed no significant differences between the thresholds of the same paw or that of the animal (labelled as either paw) measured at various time points (ANOVA followed by Newman – Keuls test) and between the threshold of the left and right paws measured at any time point (Student's *t*-test for unpaired samples).

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

Each of these analgesics caused an elevation of the noxious heat threshold of rats in a dose-dependent manner (Figure 2). As their solvents failed to cause a significant effect, the thresholds before and after drug treatment were compared with Student's *t*-test for paired samples. The minimum effective doses (the lowest applied dose causing a statistically significant elevation of the heat threshold) as well as the $ED_{1°C}$ values (the dose corresponding to a 1°C increase of the heat threshold) are shown in Table 1.

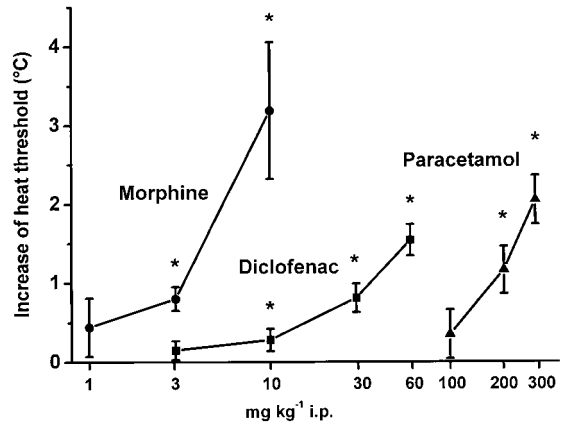

Figure 2 Effect of morphine, diclofenac or paracetamol pretreatment (i.p., 30 min) on the noxious heat threshold. Increase of threshold refers to the difference between the postdrug and the control thresholds. Data are means with s.e.m. of 6-12 animals. As all solvents failed to significantly alter the heat threshold, the postdrug threshold was compared to the control using Student's *t*-test for paired samples. Asterisks indicate statistically significant ($P<0.05$) drug effects.

Acute effect of RTX on the heat threshold

After an intraplantar injection of RTX, an acute nocifensive response consisting of licking, biting and lifting of the injected paw was observed in the rats. These reactions usually lasted for less than 5 min, although in rare cases some of these behavioural signs indicative of nociception were observed later as well. When testing the RTX-treated animals on the ITHP, lifting of the treated hindpaw was the first heat-evoked behavioural reaction on almost every occasion, which was rarely seen under control conditions. In some cases the paw-lifting reaction was phasic, lasting for about 1 s and appearing repetitively with the animal placing no weight on the treated paw between liftings, whereas in other cases a sustained lifting was observed. 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 (Figure 3). On an average, at each time point of measurement, lifting of the paw appeared at considerably (4 – 6°C) lower temperatures than paw licking. The threshold 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. The nontreated hindpaw showed only the paw-licking reaction with no change in the threshold compared to the control.

The great degree and short duration of the RTX-induced threshold drop explain why the threshold measurement protocol was altered in these experiments in the sense that the starting temperature was decreased below 15°C and the heating rate was increased from 6 to 12°C min^{-1}. This latter alteration provided shorter measurement times and allowed threshold determinations at every 5 min. The solvent of RTX (0.03% ethanol in saline, 100 μl) injected intraplantarly failed to evoke any nocifensive reaction. Upon measurement with the ITHP, no paw-lifting reaction was

Table 1 Effective doses of morphine, diclofenac and paracetamol which induce an elevation of the heat threshold or inhibit the RTX-induced threshold drop

	Morphine		*Diclofenac*		*Paracetamol*	
Elevation of the heat threshold	Minimum effective dose ($mg\,kg^{-1}$)	$ED_{1°C}$ ($mg\,kg^{-1}$)	Minimum effective dose ($mg\,kg^{-1}$)	$ED_{1°C}$ ($mg\,kg^{-1}$)	Minimum effective dose ($mg\,kg^{-1}$)	$ED_{1°C}$ ($mg\,kg^{-1}$)
	3	4.5	10	40	200	160
Inhibition of the RTX-induced threshold drop	Minimum effective dose ($mg\,kg^{-1}$)	ED_{50} ($mg\,kg^{-1}$)	Minimum effective dose ($mg\,kg^{-1}$)	ED_{50} ($mg\,kg^{-1}$)	Minimum effective dose ($mg\,kg^{-1}$)	ED_{50} ($mg\,kg^{-1}$)
	1	0.8	1	0.7	100	120
	1	0.5	1	0.6	100	115

Minimum effective dose: the minimum applied dose causing a significant increase of the heat threshold or a significant inhibition of the RTX-induced threshold drop. $ED_{1°C}$: the dose corresponding to a 1°C elevation of the heat threshold. ED_{50}: the dose corresponding to 50% of the maximal inhibition of RTX-induced threshold drop. These values were calculated on the basis of either the threshold drop at 5 min (upper value) or the sum of threshold drops at 5, 10, 15 and 20 min (lower value).

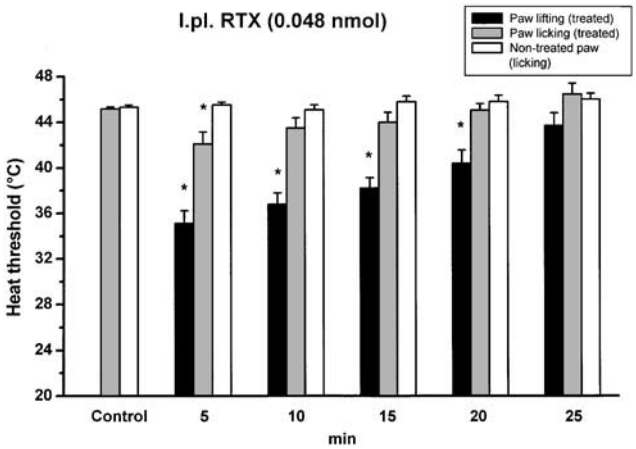

Figure 3 Effect of unilateral intraplantar injection of RTX (0.048 nmol per paw) on the heat threshold. Data are means with s.e.m. of 12 animals. Asterisks indicate values significantly different from the control threshold of the treated paw ($P<0.05$, calculated with ANOVA followed by Newman – Keuls test). The solvent of RTX (100 μl 0.03% ethanol in saline) failed to alter the heat threshold and only the paw-licking reaction was observed in the vehicle-treated animals (data not shown).

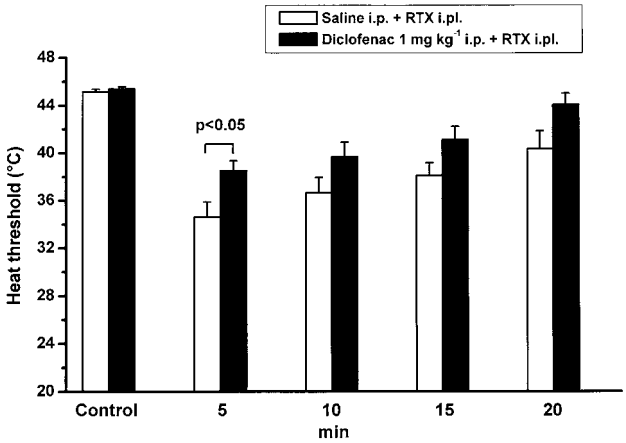

Figure 4 Effect of diclofenac pretreatment (1 $mg\,kg^{-1}$ i.p., 25 min before RTX injection) as compared to its solvent on the RTX-induced drop of heat threshold. Data are means with s.e.m. of thresholds of the RTX-treated paw ($n=12$ in both groups). The effect of diclofenac was significant ($P<0.05$, Student's *t*-test for unpaired samples) comparing the threshold drop at 5 min (as shown) or the sum of threshold drops at 5, 10, 15 and 20 min in the solvent-*versus* drug-treated animals.

observed and the heat threshold for the licking reaction of the injected paw was not different from its control at any time point of measurement.

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

Morphine, diclofenac and paracetamol inhibited the RTX-induced drop of heat threshold as compared to their solvent controls at all time points of measurement. Figure 4 shows the effect of 1 $mg\,kg^{-1}$ diclofenac as a representative example. For a quantitative assessment of the inhibitory effect of analgesics on the RTX-induced heat threshold drop, two parameters were used: the threshold drop observed 5 min after RTX administration, which represents the peak effect of RTX, and the sum of threshold drops measured at 5, 10, 15 and 20 min, which is an integral measure of the RTX action. The inhibitory effect of drugs on the RTX-induced threshold drop was expressed as percentage inhibition according to the following formula: $(Drop_{solv}-Drop_{drug})/Drop_{solv}\times 100$, where $Drop_{solv}$ and $Drop_{drug}$ refer to the average of the RTX-induced threshold drops (either at 5 min or the sum of drops) measured in the solvent- and drug-treated animals, respectively. On the basis of the percentage inhibition calculated with either parameter, all analgesics had a dose-dependent inhibitory effect (Figure 5). The minimum effective doses (the lowest applied dose causing a statistically significant inhibition of the RTX-induced threshold drop) and the ED_{50} values (the dose corresponding to 50% of the maximal inhibition of RTX-induced threshold drop) are shown in Table 1. For each drug, the dose – response curves obtained with the two calculations looked very similar, the minimum effective doses being the very same. Morphine and paracetamol showed a steep dose – response curve and a complete blockade of the RTX-evoked threshold drop was achieved by 10 and 300 $mg\,kg^{-1}$, respectively. On the contrary, diclofenac was less efficacious, the maximal inhibition of the

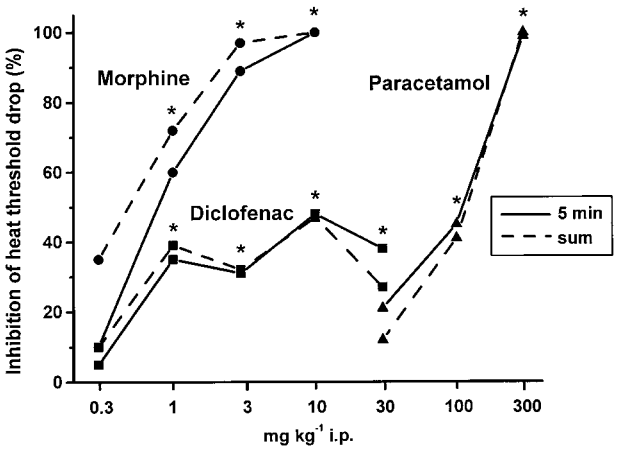

Figure 5 Dose – response relation for the inhibitory effect of morphine, diclofenac or paracetamol pretreatment (i.p., 25 min before RTX injection) on the RTX-induced drop of heat threshold. Percentage inhibition of threshold drop was calculated with the formula $(Drop_{solv}-Drop_{drug})/Drop_{solv} \times 100$, where $Drop_{soiv}$ and $Drop_{drug}$ refer to the average of threshold drops measured in the solvent- and drug-treated animals, respectively ($n = 10 - 12$ in each group). Solid lines show percentage inhibition values calculated on the basis of threshold drop 5 min after RTX injection, whereas dashed lines indicate those determined with the sum of threshold drops at 5, 10, 15 and 20 min. Asterisks indicate statistically significant ($P<0.05$) drug effects as calculated by comparisons to their solvents with Student's *t*-test for unpaired samples. The asterisks refer to points of both lines in case of each drug.

RTX effect being only around 40% and its dose – response curve was flat.

Long-term effect of RTX on the noxious heat threshold

Bilateral intraplantar administration of RTX (0.048 nmol per paw) in rats also caused an acute nocifensive reaction characterized by licking, biting and lifting of the hindpaws for less than 5 min. Subsequent heat threshold determinations with the ITHP revealed that initially a similar threshold drop was induced as in the case of unilateral RTX injection, the paw-lifting thresholds being 37.4 ± 1.5°C (5 min), 38.4 ± 1.2°C (10 min), 38.3 ± 1.1°C (15 min), 40.3 ± 1.2°C (20 min), 44.3 ± 1.3°C (25 min); $n = 12$ for each time point. An increase of the heat threshold was observed when the animals were tested 1 h after the injection (Figure 6). At this time and thereafter, the paw-lifting reaction to noxious heat did not occur any more, licking of the paw being the only nocifensive behaviour seen. The heat threshold-elevating effect of RTX was manifest as early as 1 h after administration, reached a maximum of 2.9 ± 0.5°C increase and lasted for 5 days (Figure 6). From day 6, the heat threshold remained in the control range. The solvent of RTX failed to exert any effect on the heat threshold.

Effect of I-RTX on the noxious heat threshold of control and sensitized rats

Following I-RTX treatment (0.1 and 1 nmol per paw, $n = 8$) no nocifensive behaviour was observed and no alteration of the heat threshold was measured with the ITHP either acutely (5 – 60 min) or in the long term during a follow-up of 5 days (data not shown).

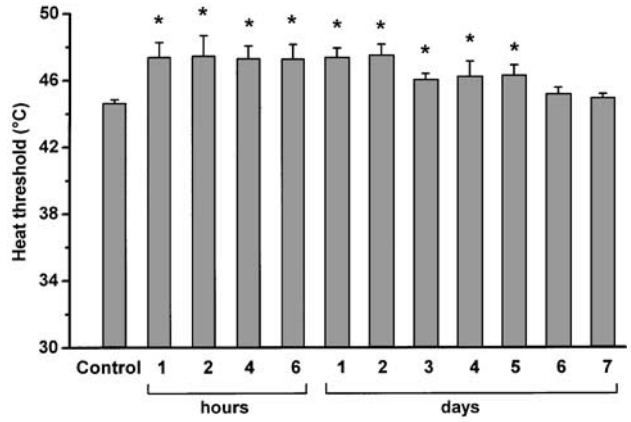

Figure 6 Long-term effect of bilateral intraplantar injection of RTX (0.048 nmol per paw) on the noxious heat threshold. Data are means with s.e.m. of 12 animals. Asterisks indicate values significantly different from control ($P<0.05$, as calculated with ANOVA followed by Newman – Keuls test). The solvent of RTX (100 μl 0.03% ethanol in saline) applied also bilaterally failed to evoke any significant changes of threshold (data not shown).

Intraplantar pretreatment 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% (threshold drop at 5 min) and 64% (sum of threshold drops) ($n = 8$, $P<0.01$ for both, Figure 7). Intraplantar 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 ($n = 8$, $P<0.05$ for both). At later time points no significant threshold alteration was measured. Intraplantar pretreatment with the P2 purinergic receptor antagonist PPADS (0.15 μmol, 5 min before) reduced the threshold-lowering effect of α,β-meATP, the percentage inhibition being 83% at 5 min ($n = 8$, $P<0.01$, Figure 7). I-RTX pretreatment (0.05 nmol per paw, 5 min before) failed to inhibit the α,β-methylene-ATP-induced drop of the heat threshold, thresholds being at 5 min 40.39 ± 1.1°C in the solvent-treated and 39.19 ± 1.1°C in the I-RTX-pretreated group ($n = 8$, $P = 0.48$, Figure 7).

Comparison of the noxious heat threshold of untreated $VR1^{+/+}$ and $VR1^{-/-}$ mice

No statistically significant difference was revealed when comparing the noxious heat threshold of untreated mice lacking the gene for VR1 receptor (45.6 ± 0.5°C) with that of their wild-type counterparts (45.2 ± 0.4°C) ($n = 10$ for both, $P = 0.76$, Student's *t*-test for unpaired samples).

Discussion

The newly developed ITHP equipment used in the present study proved to be a reliable tool for the measurement of noxious heat threshold temperature and its modulation by drugs in conscious unrestrained rats. An important feature of the ITHP method is the excellent reproducibility. The noxious heat threshold of rats remained constant upon repeated measurements at various intervals ranging from 5 min to 1 day. It is in sharp contrast to the data obtained by measuring the reaction latency with the conventional constant-tempera-

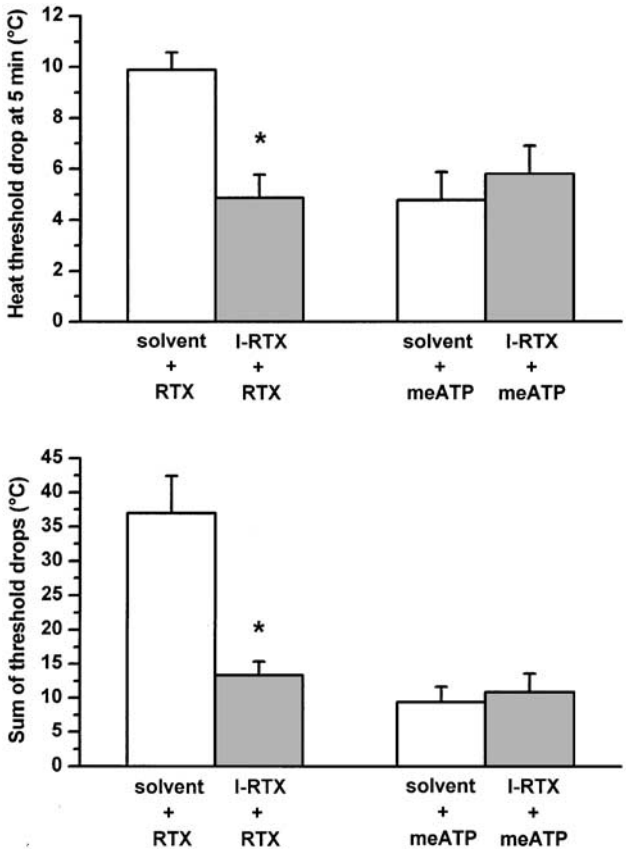

Figure 7 Effect of I-RTX (0.05 nmol per paw) on the RTX (0.048 nmol per paw)- induced and α-β-methylene-ATP (meATP, 0.3 μmol per paw)-induced drop of heat threshold. Shown are heat threshold values measured 5 min after RTX or meATP injection in the solvent- and I-RTX-pretreated animals (upper panel) as well as the sum of threshold drops (at 5, 10, 15 and 20 min for RTX; 5 and 10 min for meATP) during the allodynic/hyperalgesic response induced by RTX or meATP (lower panel). Data are means with s.e.m. of 8 animals. Asterisks indicate values significantly different compared to solvent pretreatment ($P<0.05$, as calculated with Student's *t*-test for unpaired samples).

ture hot plate method where markedly diminished latencies were characteristic upon repeated measurements (Gamble & Milne, 1989; Plone *et al*., 1996; Sandkühler *et al*., 1996). The lack of signs of either sensitization or desensitization in the ITHP test might be attributed to the fact that in this case threshold stimuli were applied which – according to their nature – evoke a smaller degree of nociceptor excitation than the suprathreshold ones used in the conventional hot plate test. 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 thresholds such as the behavioural mechanonociceptive threshold determined using von Frey hairs or the Randall – Selitto method and to heat or mechanical thresholds measured by single-unit recordings or in patch-clamp experiments on isolated primary sensory neurons.

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. These results with morphine and paracetamol are similar to those obtained by Hunskaar *et al*. (1986). It is widely accepted that the classical constant-temperature hot plate test can only reveal the antinociceptive effect of opioid analgesics, being largely insensitive to cyclooxygenase inhibitors (see Vogel & Vogel, 1997; Le Bars *et al*., 2001). In particular, diclofenac up to 100 mg kg^{-1} i.p. was found ineffective in the classical hot plate test in all studies published (Bjorkman *et al*., 1990; Engelhardt *et al*., 1995; Santos *et al*., 1998), whereas for paracetamol ambiguous results have been provided (Pini *et al*., 1996; Amabeoku *et al*., 2000; Kouadio *et al*., 2000; Sandrini *et al*., 2001). The minimum effective dose of morphine determined in the present study is similar to that obtained for the drug with the constant-temperature hot plate test (see Vogel & Vogel, 1997; Le Bars *et al*., 2001).

The present study determined for the first time the real heat threshold-lowering action of a VR1 receptor agonist in conscious unrestrained animals. In our earlier studies in which the hindpaw of the rat was immersed into a water bath of different temperatures, only an approximate noxious heat threshold measurement could be made to follow the acute effect of systemic capsaicin pretreatment (Szolcsányi, 1990). 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. Interestingly, intraplantar injection of RTX induced a behavioural reaction on the hot plate rarely encountered in untreated rats. In the RTX-treated rats, lifting of the paw was the first nocifensive response at each time point of heat threshold measurement, followed by paw licking with a shorter or longer delay at a higher temperature. In cases when paw lifting was sustained, lasting for several seconds, the injected paw was not in contact with the plate, which obviously hindered the occurrence of the licking reaction. For these reasons, the paw-lifting reaction was used as an end point. It is interesting to note that the end point seen in the ITHP test in untreated rats (paw licking) is the same as typically observed in the conventional hot plate test, while the reaction characterizing RTX allodynia (paw lifting) resembles the end point of the plantar test employing stimulation of the paw with radiant heat (Hargreaves *et al*., 1988). The reason for this is unknown; maybe the sensitized state induced by RTX alters the central processing of signals evoked by heat stimuli in a way that a different behavioural response pattern is generated. The threshold drop induced by RTX was confined to the treated paw and was very pronounced, thresholds being in the innocuous range of 34 – 41°C for the whole period during which the threshold was significantly lower than the control. Therefore this response can be designated as a heat allodynia. In the conventional thermal hyperalgesia models employing reflex latency measurements the distinction between hyperalgesia and allodynia is difficult as a latency shortening cannot be considered as being noxious or innocuous. The threshold drop induced by RTX was caused by the activation of VR1 receptors as the heat allodynia was inhibited by the locally applied VR1 receptor antagonist I-RTX.

The heat-sensitizing effect of the VR1 receptor agonist capsaicin is well known. In humans, capsaicin applied topically to skin causes heat allodynia by lowering the heat threshold to 30 – 31°C (Szolcsányi, 1977) and induces hyperalgesia after intradermal injection (Simone *et al*., 1987). Intraplantar or topical application of capsaicin in rats evokes heat hyperalgesia as revealed by a reduction of paw-withdrawal latency (Gilchrist *et al*., 1996; Yeomans *et al*., 1996). The heat-

sensitizing effect of capsaicin was observed on isolated sensory neurons in patch-clamp experiments as well as in single-unit studies (Kirschstein *et al*., 1997; Günther *et al*., 1999; Vyklicky *et al*., 1999). In the latter study on isolated sensory neurons, a threshold drop even below the ambient temperature was noticed. The present results fit well to these data indicating that RTX, another VR1 receptor agonist, leads to a marked drop of the heat threshold even to the innocuous range.

Morphine, diclofenac and paracetamol exerted a dose-dependent inhibitory effect on the RTX-induced threshold drop. The two methods of calculation used for quantification of this antiallodynic effect of analgesics provided congruent results (Figure 5) indicating that both parameters (threshold drop at 5 min or the sum of threshold drops) are equally suitable for measuring the antinociceptive action of drugs in this model. The phasic nature, that is, the fast onset and short duration of the RTX-induced threshold drop, makes it unlikely that a significant inflammatory reaction develops during RTX allodynia. Therefore, the ability of diclofenac to inhibit this response probably reflects the antinociceptive rather than anti-inflammatory effect of the drug. By contrast, in several hyperalgesia tests commonly used, such as those employing carrageenan or complete Freund's adjuvant, an inflammatory reaction develops that makes difficult to separate the antinociceptive and the anti-inflammatory effects of drugs (Vogel & Vogel, 1997). It is worth emphasizing that the minimum effective doses for the antiallodynic effect were smaller than those for elevation of the control heat threshold in case of all three analgesics studied. It means that inhibition of the RTX-induced threshold drop is a more sensitive model to reveal the thermal antinociceptive effect of these drugs than the elevation of the control heat threshold. As the minimum effective dose of morphine and diclofenac for inhibition of RTX-induced heat allodynia is very close to their standard analgesic doses used in humans, this model complies with another important requirement raised towards sensitive nociceptive tests.

Measurement of the noxious heat threshold with the ITHP proved to be suitable also to reveal the long-term, but still reversible, local sensory desensitizing effect of RTX similarly as described previously for capsaicin using a paw-immersion method (Szolcsányi, 1985; 1987b). In previous studies using the constant-temperature hot plate and the tail-flick test, the thermal antinociceptive effect of capsaicin was revealed in some studies but not in others (Szolcsányi, 1976; 1985; Obál *et al*., 1979; Hayes & Tyers, 1980; Gamse, 1982; Bittner & Lahann, 1984), while its desensitizing effect was unequivocally shown by electrophysiological recordings (Szolcsányi, 1987a, 1990). In contrast to the conventional methods, the excellent reproducibility upon daily measurements of the heat threshold in the ITHP test is another factor that makes this method suitable for the long-term investigation of noxious heat responsiveness.

In agreement with *in vitro* data (Wahl *et al*., 2001; Undem & Kollarik, 2002) I-RTX does not appear to have a partial agonistic activity at the VR1 receptor as indicated by the lack of any nocifensive reaction or an acute heat threshold-lowering effect up to 10 μM concentration following intraplantar injection. 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. α,β-meATP, a metabolically stable analogue of adenosine 5′-triphosphate, also evoked a drop of the noxious heat threshold that was smaller both in magnitude and duration compared to the effect of RTX. The α,β-meATP-induced threshold drop was inhibited by the P2 purinergic receptor antagonist PPADS indicating that P2 receptor activation was involved. These results are in accordance with the thermal hyperalgesic effect of ATP and its analogues observed using the plantar test based on latency measurement (Hamilton *et al*., 1999). The heat threshold-lowering effect of α,β-meATP was used as an internal control for the selectivity of I-RTX in the present model. As I-RTX failed to inhibit the effect of α,β-meATP, at a concentration producing a substantial inhibition of the RTX-induced drop of heat threshold, it is very unlikely that I-RTX exerted a nonspecific, VR1 receptor-unrelated (e.g. local anaesthetic) effect at the applied dose. This is also supported by the finding that I-RTX had no effect on the control heat threshold. These data show that I-RTX behaves as a potent and selective VR1 receptor antagonist devoid of measurable partial agonistic activity and liability to be converted to RTX also under *in vivo* conditions, at least in the present model. The results obtained with I-RTX also exemplify the advantage of the ITHP test that both acute and long-term measurements can reliably be performed because of the high stability of heat threshold.

The lack of an effect of I-RTX on the control heat threshold is compatible with the hypothesis that the VR1 receptor, which is the first identified noxious heat-sensing ion channel, is not involved in signalling the noxious heat threshold temperature of peripheral nociceptors. Indeed, recent behavioural studies conducted in mice lacking the gene for the VR1 receptor showed no impairment of thermonociception up to 48 – 50°C stimulation intensity as measured by the conventional hot plate, tail-flick and plantar tests (Caterina *et al*., 2000; Davis *et al*., 2000). In accordance with this, in the present study the noxious heat threshold of the VR1 receptor knockout mice was found not different from that of their wild-type counterparts indicating that the VR1 channels were not involved in the behavioural determination of the noxious heat threshold of peripheral nociceptors under physiological conditions. A possible candidate for being the major noxious heat-sensing ion channel in peripheral nociceptors is the recently cloned VRL-3/TRPV3 receptor (Xu *et al*., 2002; Smith *et al*., 2002).

In conclusion, the noxious heat threshold of untreated rats measured by a new ITHP was 45.3 ± 0.3°C. Repeated measurements in rats at intervals ranging from 5 min to 1 day were reproducible making the method suitable for measuring the antinociceptive effect of morphine, diclofenac and paracetamol as well as the lasting reversible sensory desensitizing effect of the VR1 receptor agonist RTX. A heat allodynia model based on the RTX-induced profound threshold drop was described, which is a sensitive method for measuring the effect of opioid and nonopioid analgesics. Using the ITHP method, I-RTX was found to be a potent and selective VR1 receptor antagonist *in vivo* upon intraplantar administration. The VR1 receptor is unlikely to be involved in the determination of the behavioural noxious heat threshold of peripheral nociceptors. All these findings show that the ITHP test is a useful method and it has several advantages over the conventional hot plate test currently used for screening drugs for analgesic actions.

This study was supported by Hungarian Grants OTKA T-034911, OTKA T-037523, OTKA TS-040753, ETT 04-367/2000, NRDP1/047/2001, NRDP1A/0021/2002 and by the Hungarian Academy of Sciences. G. Pethö was supported by the Bolyai János Research Fellowship. Special thanks to Dr Róbert Pórszász for participating in the development and technical validation of the increasing-temperature hot plate equipment as well as to Katalin Keserü for expert technical assistance. We are grateful to Dr John B. Davis, GlaxoSmithKline, Harlow, U.K. for providing the VR1 receptor knockout mice.

References

AMABEOKU, G.J., GREEN, I., EAGLES, P. & BENJEDDOU, M. (2000). Effects of *Tarchonantus camphoratus* and *Eriocephalus africanus* on nociception in mice and pyrexia in rats. *Phytomedicine,* **7,** 517 – 522.

BITTNER, M.A. & LAHANN, T.R. (1984). Biphasic time-course of capsaicin-induced substance P depletion: failure to correlate with thermal analgesia in the rat. *Brain Res.,* **322,** 305 – 309.

BJORKMAN, R., HEDNER, J., HEDNER, T. & HENNING, M. (1990). Central, naloxone-reversible antinociception by diclofenac in the rat. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **342,** 171 – 176.

CATERINA, M.J., LEFFLER, A., MALMBERG, A.B., MARTIN, W.J., TRAFTON, J., PETERSEN-ZEITZ, K.R., KOLTZENBURG, M., BASBAUM, A.I. & JULIUS, D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science,* **288,** 306 – 313.

CATERINA, M.J., SCHUMACHER, M.A., TOMINAGA, M., ROSEN, T.A., LEVINE, J.D. & JULIUS, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature,* **389,** 816 – 824.

DAVIS, J.B., GRAY, J., GUNTHORPE, M.J., HATCHER, J.P., DAVEY, P.T., OVEREND, P., HARRIES, M.H., LATCHMAN, J., CLAPHAM, C., ATKINSON, K., HUGHES, S.A., RANCE, K., GRAU, E., HARPER, A.J., PUGH, P.A., ROGERS, D.C., BINGHAM, S., RANDALL, A. & SHEARDOWN, A.S. (2000). Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature,* **405,** 183 – 187.

ENGELHARDT, G., HOMMA, D., SCHLEGEL, K., UTZMANN, R. & SCHNITZLER, C. (1995). Anti-inflammatory, analgesic, antipyretic and related properties of meloxicam, a new non-steroidal anti-inflammatory agent with favorable gastrointestinal tolerance. *Inflamm. Res.,* **44,** 423 – 433.

GAMBLE, G.D. & MILNE, R.J. (1989). Repeated exposure to sham testing procedures reduces reflex withdrawal and hot-plate latencies: attenuation of tonic descending inhibition? *Neurosci. Lett.,* **96,** 312 – 317.

GAMSE, R. (1982). Capsaicin and nociception in the rat and mouse. Possible role of substance P. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **320,** 205 – 216.

GILCHRIST, H.D., ALLARD, B.L. & SIMONE, D.A. (1996). Enhanced withdrawal responses to heat and mechanical stimuli following intraplantar injection of capsaicin in rats. *Pain,* **67,** 179 – 188.

GÜNTHER, S., REEH, P.W. & KRESS, M. (1999). Rises in $[Ca^{2+}]_i$ mediate capsaicin- and proton-induced heat sensitization of rat primary nociceptive neurons. *Eur. J. Neurosci.,* **11,** 3143 – 3150.

HAMILTON, S.G., WADE, A. & MCMAHON, S.B. (1999). The effects of inflammation and inflammatory mediators on nociceptive behaviour induced by ATP analogues in the rat. *Br. J. Pharmacol.,* **126,** 326 – 332.

HARGREAVES, K., DUBNER, R., BROWN, F., FLORES, C. & JORIS, J. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain,* **32,** 77 – 88.

HAYES, A.G. & TYERS, M.B. (1980). Effects of capsaicin on nociceptive heat, pressure and chemical thresholds and on substance P levels in the rat. *Brain Res.,* **189,** 561 – 564.

HUNSKAAR, S., BERGE, O-G. & HOLE, K. (1986). A modified hot-plate test sensitive to mild analgesics. *Behav. Brain Res.,* **21,** 101 – 108.

KIRSCHSTEIN, T., BUSSELBERG, D. & TREEDE, R.D. (1997). Coexpression of heat-evoked and capsaicin-evoked inward currents in acutely dissociated rat dorsal root ganglion neurons. *Neurosci. Lett.,* **231,** 33 – 36.

KOUADIO, F., KANKO, C., JUGE, M., GRIMAUD, N., JEAN, A., N'GUESSAN, Y.T. & PETIT, J.Y. (2000). Analgesic and antiinflammatory activities of an extract from Parkia biglobosa used in traditional medicine in the Ivory Coast. *Phytother. Res.,* **14,** 635 – 637.

LE BARS, D., GOZARIU, M. & CADDEN S.W. (2001). Animal models of nociception. *Pharmacol. Rev.,* **53,** 597 – 652.

OBÁL, F., BENEDEK, G., JANCSÓ-GÁBOR, A. & OBÁL, F. (1979). Salivary cooling, escape reaction and heat pain in capsaicin-desensitized rats. *Pflügers Arch.,* **382,** 249 – 254.

PINI, L.A., SANDRINI, M. & VITALE, G. (1996). The antinociceptive action of paracetamol is associated with changes in the serotonergic system in the rat brain. *Eur. J. Pharmacol.,* **308,** 31 – 40.

PLONE, M.A., EMERICH, D.F. & LINDNER, M.D. (1996). Individual differences in the hotplate test and effects of habituation on sensitivity to morphine. *Pain,* **66,** 265 – 270.

SANDKÜHLER, J., TREIER, A.C., LIU, X.G. & OHNIMUS, M. (1996). The massive expression of c-fos protein in spinal dorsal horn neurons is not followed by long-term changes in spinal nociception. *Neuroscience,* **73,** 657 – 666.

SANDRINI, M., ROMUALDI, P., CAPOBIANCO, A., VITALE, G., MORELLI G., PINI, L.A. & CANDELETTI, S. (2001). The effect of paracetamol on nociception and dynorphin A levels in the rat brain. *Neuropeptides,* **35,** 110 – 116.

SANTOS, A.R., VEDANA, E.M. & DE-FREITAS, G.A. (1998). Antinociceptive effect of meloxicam, in neurogenic and inflammatory nociceptive models in mice. *Inflamm. Res.,* **47,** 302 – 307.

SIMONE, D.A., NGEOW, J.Y.F., PUTTERMAN, G.J. & LAMOTTE, R.H. (1987). Hyperalgesia to heat after intradermal injection of capsaicin. *Brain Res.,* **418,** 201 – 203.

SMITH, G.D., GUNTHORPE, M.J., KELSELL, R.E., HAYES, P.D., REILLY, P., FACER, P., WRIGHT, J.E., JERMAN, J.C., WALHIN, J.P., OOI, L., EGERTON, J., CHARLES, K.J., SMART, D., RANDALL, A.D., ANAND, P. & DAVIS, J.B. (2002). TRPV3 is a temperature-sensitive vanilloid receptor-like protein. *Nature,* **418,** 186 – 190.

SZÁLLÁSI, Á. & BLUMBERG, P.M. (1999). Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol. Rev.,* **51,** 159 – 212.

SZOLCSÁNYI, J. (1976). On the specificity of pain producing and sensory neuron blocking effect of capsaicin. In: *Symposium on Analgesics*. ed. Knoll, J. & Vizi, E.S. pp. 167 – 172. Budapest: Akadémiai Kiadó.

SZOLCSÁNYI, J. (1977). A pharmacological approach to elucidation of the role of different nerve fibres and receptor endings in mediation of pain. *J. Physiol. (Paris),* **73,** 251 – 259.

SZOLCSÁNYI, J. (1985). Sensory receptors and the antinociceptive effects of capsaicin. In: *Tachykinin Antagonists*. ed. Hakanson, R. & Sundler, F. pp. 45 – 54. Amsterdam: Elsevier.

SZOLCSÁNYI, J. (1987a). Selective responsiveness of polymodal nociceptors of the rabbit ear to capsaicin, bradykinin and ultraviolet irradiation. *J. Physiol. (London),* **388,** 9 – 23.

SZOLCSÁNYI, J. (1987b). Capsaicin and nociception. *Acta Physiol. Hung.,* **69,** 323 – 332.

SZOLCSÁNYI, J. (1990). Capsaicin, irritation and desensitization: neurophysiological basis and future perspectives. In: *Chemical Senses. Vol. 2. Irritation*. ed. Green, B.G., Mason, J.R. & Kare, M.R. pp. 141 – 168. New York: Marcel Dekker.

SZOLCSÁNYI, J., PETHÖ, G., SZÖKE, É., ALMÁSI, R. & SERESS, L. (2001). Effect of resiniferatoxin, anandamide and analgesics on noxious heat threshold. *Proceedings of the Society for Neuroscience*, San Diego, Program No. 926.10.

TOMINAGA, M., CATERINA, M.J., MALMBERG, A.B., ROSEN, T.A., GILBERT, H., SKINNER, K., RAUMANN, B.E., BASBAUM, A.I. & JULIUS, D. (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron,* **21,** 531 – 543.

UNDEM, B.J. & KOLLARIK, M. (2002). Characterization of the vanilloid receptor 1 antagonist iodo-resiniferatoxin on the afferent and efferent function of vagal sensory C-fibers. *J. Pharmacol. Exp. Ther.,* **303,** 716 – 722.

VOGEL, H.G. & VOGEL W.H. (1997). *Drug Discovery and Evaluation. Pharmacological Assays*. Berlin/Heidelberg/New York: Springer.

VYKLICKY, L., VLACHOVÁ, V., VITASKOVÁ, Z., DITTERT, I., KABAT, M. & ORKAND, R.K. (1999). Temperature coefficient of membrane currents induced by noxious heat in sensory neurones in the rat. *J. Physiol. (London),* **517,** 181 – 192.

WAHL, P., FOGED, C., TULLIN, S., & THOMSEN, C. (2001). Iodoresiniferatoxin, a new potent vanilloid receptor antagonist. *Mol. Pharmacol.,* **59,** 9 – 15.

XU, H., RAMSEY, I.S., KOTECHA, S.A., MORAN, M.M., CHONG, J.A., LAWSON, D., GE, P., LILLY, J., SILOS-SANTIAGO, I., XIE, Y., DISTEFANO, P.S., CURTIS, R. & CLAPHAM, D.E. (2002). TRPV3 is a calcium-permeable temperature-sensitive cation channel. *Nature,* **418,** 181 – 186.

YEOMANS, D.C., PIREC, V. & PROUDFIT, H.K. (1996). Nociceptive responses to high and low rates of noxious cutaneous heating are mediated by different nociceptors of the rat: behavioral evidence. *Pain,* **68,** 133 – 140.

ZIMMERMANN M. (1983). Ethical guidelines for investigations of experimental pain in conscious animals. *Pain,* **16,** 109 – 110.

(Received September 8, 2002
Revised January 8, 2003
Accepted February 10, 2003)