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Pharmacological effect of capsaicin on rat avoidance behaviours elicited by sine-wave electrical stimulation of different frequencies by Neurometer

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Abstract

The Neurometer is a diagnostic device for measuring the perception and threshold of transcutaneous stimulation. It has been used in patients to selectively activate Aβ-, Aδ- and C-fibres in the primary afferents at different stimulus frequencies (2000, 250 and 5 Hz, respectively). In this study, we investigated use of the Neurometer to selectively activate nerves in conscious rats. The behavioural endpoint of paw withdrawal was used to measure the current threshold (CT). This behaviour was elicited by a lower stimulus current than other behaviours evoked by Neurometer stimulation and caused only mild stress in rats. Repeated topical application of capsaicin (four doses of 100 μg) or systemic administration of the capsaicin analogue resiniferatoxin (30 or 300 μg kg^{-1}) increased the CT value for this behaviour at 5 Hz stimulation but not at 2000 Hz or 250 Hz. This change in CT at 5 Hz is probably due to C-fibre desensitization by the pharmacological treatments. The combination of 5 Hz sine-wave stimulation with a Neurometer and the observation of paw withdrawal behaviour make it possible to perform preclinical studies of C-fibres in animals as an alternative to the use of high- and low-rate heating of the paw.

Introduction

A method for transcutaneous electrical stimulation using a Neurometer has been developed and used to measure both sensory perception and pain thresholds, in order to quantify nerve dysfunction in patients with neuropathic pain or various other diseases (Raj et al 2001; Yamashita et al 2002; Matsumoto et al 2006; Passavanti et al 2006; Sakai et al 2006). In human studies, sine-wave electrical stimulation with the Neurometer at three different frequencies of (2000, 250 and 5 Hz) selectively activates the primary afferents of large myelinated Aβ-fibres, small myelinated Aδ-fibres and unmyelinated C-fibres, respectively (Masson et al 1989; Baquis et al 1999).

Assessment of current threshold (CT) at the three different frequencies has also been used in pharmacological studies in animals (Kiso et al 2001; Akada et al 2005; Oda et al 2005). The relationship between the stimulus frequency generated by the Neurometer and the subtypes of primary afferents activated in human studies has been extrapolated to animal studies. However, the relationship in animals remains to be clarified if rational pharmacological studies are to be performed. One report has attempted to analyse the relationship in ex-vivo preparations and in anaesthetized rats using electrophysiological methods (Koga et al 2005). Clarification of the relationship in conscious rats would not only support the validity of the methodology in animal behavioural studies as an alternative approach to selective activation of nerve fibres, which has been performed using high and low heating rates (Yeomans & Proudfit 1996; Yeomans et al 1996), but would also be useful to define the neurophysiological rationale for clinical use of the device.

In this paper, we have first defined a behavioural approach to the measurement of CT in conscious rats. We have also studied the effects of capsaicin and its analogue resiniferatoxin (RTX) on the behaviour elicited by three different stimulus frequencies, to test the selectivity of nerve activation by the Neurometer. Our results indicate that the Neurometer is indeed a useful tool for selective activation of nerve fibres in both physiological and pharmacological

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studies in conscious rats, and is particularly effective in investigations focusing on C-fibres.

Materials and Methods

Animals

Male Sprague–Dawley rats of international genetic standard were purchased from Charles River Laboratories (Yokohama, Japan). We used 48 rats, 10–12 weeks of age, weighing 351–442 g. Pairs of rats were housed in cages with free access to food and water and in conditions of constant temperature (23 ± 2°) and humidity (55 ± 15%), with a 12 h light–dark cycle (lights on at 07:00).

The day before the experiment, the rats' backs were shaved, and the rats were habituated to a condition of slight restraint using a Ballman cage (Natsume, Tokyo, Japan). The procedures used in this study were approved by the Animal Ethics Committee at the PGRD Nagoya Laboratories according to the Laboratory Animal Welfare guidelines.

Drugs

Both capsaicin (Wako Pure Chemical Industries, Osaka, Japan) and RTX (Sigma, St Louis, MO, USA) were dissolved in normal saline containing 10% ethanol and 10% Tween 80. A 100 μg dose of capsaicin was injected subcutaneously into the site where the stimulating electrode was attached. RTX (30 or 300 $\mu g\,kg^{-1}$) was injected subcutaneously into the back of the rats for systemic administration.

Experimental protocol

A small stimulating electrode (ATE1925, Neurotron Inc., Baltimore, MD, USA) was attached to the sole of the hindpaw, and was wrapped with tape (Softape, Neurotron Inc.) to hold it on the skin. A skin patch dispersion electrode (SDE44, Neurotron Inc.) was placed on the back of the rat. Electric conduction between the electrodes and the skin was ensured by application of an electroconductive gel (GTGL, Neurotron Inc.) to the area.

Rats with the electrodes attached were kept for at least 15 min in the Ballman cage. Transcutaneous nerve stimulation using a sine-wave current was applied to the plantar surface, using the animal response test mode of the Neurometer CPT/C (Neurotron Inc.). In this mode, the current intensity was gradually changed automatically in increments of 5 or 10 CPT units (50 or 100 μA) for a duration of 3 s. The minimum current intensity necessary to evoke any behaviour was defined as the CT. The measurement process was repeated 3–5 times at 15 min intervals.

Rats showed different behavioural responses to the first trial in each series of experiments compared with other trials. The result of the first trial was therefore discounted. The arithmetic mean value of the CT values from each trial, with the exception of the first, was defined as the CT in each animal.

To determine capsaicin-licking behaviour, the duration of the behaviour was counted in the initial 5 min period following the subcutaneous injection of 10 μg capsaicin (Sakurada et al 1992; Sawynok et al 2006).

Statistical analysis

Data are expressed as mean ± s.e.m. Comparisons between two groups were performed using the unpaired Student's *t*-test. Comparisons among three groups were performed using one-way analysis of variance followed by Dunnett's test. $P < 0.05$ was considered to be significant.

Results

Evoked behaviours with Neurometer stimulation

Transcutaneous electrical stimulation at frequencies of 2000, 250 and 5 Hz to the hindpaw induced paw withdrawal behaviour in rats at lower intensities. Rat behaviour changed progressively as the intensity increased, for all frequencies tested. At higher intensities stimulation caused hindpaw flinching, body wriggling and, ultimately, vocalization. CTs tended to differ for the different behaviours: vocalization was evoked by a significantly higher current intensity than paw withdrawal (Table 1). Based on these results, paw withdrawal was chosen as the endpoint for the measurement of CT in further experiments, in order to minimize the stress on conscious rats.

Repeated administration of capsaicin

Capsaicin (100 μg)was applied topically to the hindpaw with the stimulus electrode four times at intervals of 1 h. CT intensity was measured at 1 h and 24 h after the final application of capsaicin. Paw withdrawal behaviour tended to require a stronger current at 1 h after the final treatment at all stimulus frequencies in both the vehicle-treated and capsaicin-treated groups. The CT at 5 Hz stimulation after 1 h was $78.3 \pm 11.2 \times 10^{-5}$ A in the capsaicin group compared with $45.0 \pm 7.9 \times 10^{-5}$ A in the vehicle group (n = 6 in each group; $P < 0.05$) (Figure 1). After 24 h, the capsaicin group tended to show a higher CT than the vehicle group but the difference was not significant. There was no significant difference between the vehicle- and capsaicin-treated groups at any time point at either 2000 Hz or 250 Hz stimulation (Figure 1).

Resiniferatoxin treatment

CT intensity was measured by paw withdrawal behaviour after subcutaneous administration of 30 or 300 $\mu g\,kg^{-1}$ RTX. On the day after the administration (day 1), CT was increased significantly at 5 Hz stimulation (Figure 2). On day 1, CT was

Table 1 Comparison of paw withdrawal and vocalization in perception of current threshold

	Current threshold (10^{-5} A)		
	5 Hz	250 Hz	2000 Hz
Paw withdrawal	39.2 ± 3.8	47.9 ± 3.8	118.3 ± 6.5
Vocalization	86.3 ± 8.8***	110.8 ± 3.7***	238.3 ± 13.4***

Data are from eight rats. The current threshold to evoke each behaviour was measured in the same rats. ***$P < 0.005$ between two behaviours (Student's *t*-test).

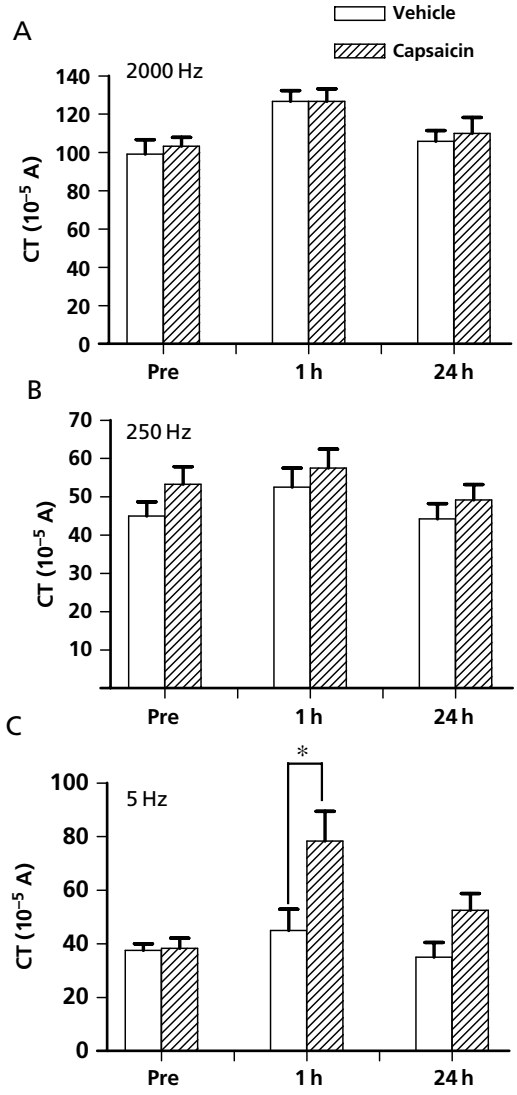

Figure 1 Effect of repeated injection of capsaicin on current threshold (CT). Capsaicin or vehicle was administered subcutaneously into the plantar surface of the hind paw every hour for 4 h. CT was measured at 1 h and 24 h after the final administration by observing paw withdrawal behaviour in response to stimulation with 2000 Hz (A), 250 Hz (B) and 5 Hz (C) stimulation. Data are from six rats in each group. $^{*}P < 0.05$ vs vehicle-treated group (Student's *t*-test).

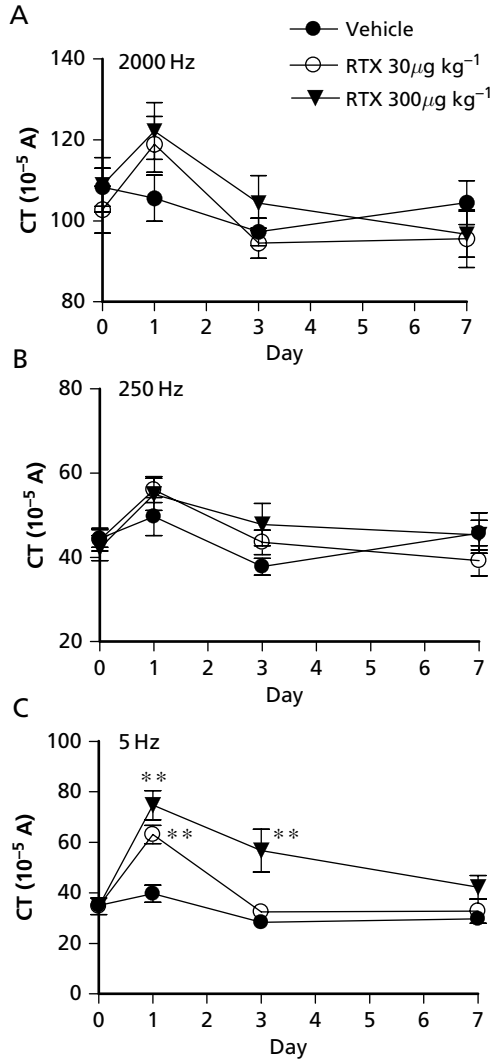

Figure 2 Change of current threshold (CT) after single subcutaneous treatment with resiniferatoxin (RTX; 30 or 300 μg kg^{-1}) or vehicle on day 0. CT was measured at days 1, 3 and 7 after treatment in response to stimulation with 2000 Hz (A), 250 Hz (B) and 5 Hz (C). Data are from six rats in each group. $^{**}P < 0.01$ vs vehicle-treated group (one-way analysis of variance followed by Dunnett's test).

$63.1 \pm 3.6 \times 10^{-5}$ A in the 30 μg kg^{-1} group and $74.7 \pm 5.8 \times 10^{-5}$ A in the 300 μg kg^{-1} group, compared with $39.7 \pm 3.4 \times 10^{-5}$ A in the vehicle-treated group (all n = 6; $P < 0.01$). The CT increase ceased on day 3 in the 30 μg kg^{-1} group but in the 300 μg kg^{-1} group a stronger current was still required to evoke the behaviour on day 3 (Figure 2). By day 7, the CT had decreased to a similar level in all three groups.

There was no significant change in CT even after 300 μg kg^{-1} RTX at 2000 Hz or 250 Hz stimulation on any day (Figure 2).

C-fibre activation in the capsaicin model has been extensively studied and is much better understood than other chemogenic pain models. It is therefore an ideal model to show C-fibre involvement. The occurrence of capsaicin-induced licking behaviour was also tested to confirm the relationship between systemic RTX treatment (300 μg kg^{-1}) and its ability to desensitize C-fibres on days 1 and 7 after RTX treatment. RTX-treated rats showed no licking behaviour on day 1 after subcutaneous injection of 10 μg of capsaicin into the hindpaw, whereas the behaviour was elicited on day 7 (Table 2). Licking times were similar in vehicle- and RTX-treated rats on day 7. Vehicle-treated rats showed licking behaviour on day 1 following the application of 10 μg capsaicin.

Discussion

We have tested transcutaneous electrical stimulation with different frequencies using a Neurometer in conscious rats. In previous animal studies, various endpoints have been used to define the CT in rats, including vocalization, startling and

Table 2 Duration of capsaicin-induced paw licking behaviour (s) in rats treated with resiniferatoxin (300 μg kg^{-1})

	Day 1	Day 7
Vehicle	27.2 ± 4.0	28.8 ± 2.6
Resiniferatoxin	0	22.1 ± 5.4

Data are from five rats in each group. Differences between the groups on day 7 are not significant.

paw withdrawal (Kiso et al 2001; Akada et al 2005; Oda et al 2005). However, vocalization requires a high stimulus intensity to be evoked. Paw withdrawal can be elicited with relatively mild electric stimulation compared with other stimulus-induced behaviours. This low-level stimulation caused little stress to the conscious rats being tested and also lessens the supraspinal emotional effects induced by intense stimulation. A recent study using a Neurometer also chose paw withdrawal as the endpoint with which to measure CT in order to minimize stress to the animals (Nagakura et al 2008). Only this behaviour should be used as the endpoint for the measurement of CT in future animal studies using Neurometers.

The CT for paw withdrawal increased only at 5 Hz stimulation, and did not change at 2000 Hz or 250 Hz stimulation after repeated capsaicin treatment or RTX administration. Repeated capsaicin administration desensitizes nociceptive C-fibres (Holzer 1991; Winter et al 1995) and inhibits C-fibre-mediated pain responses (Field et al 1999). Other desensitization effects of capsaicin on C-fibre polymodal nociceptors have also been reported (Holzer 1991). The systemic administration of RTX, an ultrapotent analogue of capsaicin, can induce C-fibre desensitization and can prolong paw withdrawal latency in response to a thermal stimulus (Szabo et al 1999).

C-fibres involved in animal behaviour are not completely desensitized by capsaicin and RTX (a limitation of all pharmacological approaches). Our results show that only 5 Hz CT was affected after capsaicin or RTX treatment, suggesting a strong correlation between C-fibre desensitization by these drugs and the inhibition of the paw withdrawal behaviour evoked by the low stimulus frequency. These pharmacological findings are consistent with the idea that a subset of unmyelinated C-fibre axons in the peripheral nerve are the source of the withdrawal response and are activated at low frequencies but less efficiently at higher stimulus frequencies (Nagakura et al 2008). Our results demonstrate C-fibre desensitization to 5 Hz stimulation by repeated application of capsaicin to the hindpaw and also by systemic administration of RTX. From these studies, it seems clear that 5 Hz stimulation activates C-fibres.

Koga and colleagues (2005) have reported that C-fibre discharge, recorded in ex-vivo preparations of dorsal root ganglion using an intracellular electrophysiological technique, was activated only by 5 Hz stimulation, despite co-activation of Aβ- and Aδ-fibres, which have lower discharge rates. This suggests that the discharge frequencies of Aβ- and Aδ-fibres elicited by 5 Hz stimulation did not reach levels sufficient to induce functional sensation. This electrophysiological presumption in ex-vivo studies agrees with our proposal that paw-withdrawal behaviour elicited by 5 Hz stimulation in conscious rats results from the functional activation of a subset of C-fibres in primary afferents.

Repeated capsaicin administration (four doses of 4575 μg) has been shown to increase CT at both 250 Hz and 5 Hz stimulation (Kiso et al 2001). In our experiment (four applications of 100 μg), CT changed at 5 Hz only. The different results at 250 Hz stimulation after repeated capsaicin administration are likely to reflect the different concentrations of capsaicin in the hindpaw. The higher dose of capsaicin used by Kiso et al may exert an additional inhibitory effect on other afferent subtypes in addition to C-fibres (e.g. decreased body temperature). A suitable capsaicin concentration for repeated administration in Neurometer studies remains to be determined.

At the whole-animal level, the behaviour evoked by 5 Hz sine-wave electrical stimulation correlates with activation of a population of afferent C-fibres in the peripheral nervous system. Combined use of 5 Hz transcutaneous stimulation and the endpoint of paw withdrawal confirmed in this study provides an alternative approach to the use of high- and low-rate heating stimulation for the study of C-fibre-mediated behaviour in animals (Yeomans 1996; Yeomans & Proudfit et al 1996).

Conclusion

Paw withdrawal is the only suitable behavioural endpoint for the measurement of CT in conscious rats in Neurometer studies in terms of animal welfare and of simple stimulation without emotional effects. Pharmacological results using capsaicin and its analogue RTX to desensitize a population of afferent C-fibres suggest that the behaviour at 5 Hz stimulation is elicited by the functional activation of C-fibres in peripheral somatic afferents. This supports the proposed paradigm of nerve selectivity in Neurometer studies at low frequencies in both clinical and non-clinical contexts. Our study has shown that the Neurometer is a useful device for selective nerve stimulation and for the quantification of sensory perception in both clinical and preclinical studies. Our findings support use of the Neurometer as a quantitative and selective peripheral nerve stimulator for use in physiological and pharmacological animal studies investigating pain, analgesic drugs and other somatic sensory functions.

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