Role of intracellular calcium in acute thermal pain perception

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Neuropharmacology 47 (2004) 935–944
NEURO PHARMACOLOGY
www.elsevier.com/locate/neuropharm

Role of intracellular calcium in acute thermal pain perception

Nicoletta Galeotti *, Alessandro Bartolini, Carla Ghelardini

Department of Preclinical and Clinical Pharmacology, University of Florence, Viale G. Pieraccini 6, 50139 Florence, Italy

Received 7 April 2004; received in revised form 11 June 2004; accepted 30 June 2004

Abstract

The role of intracellular calcium in acute thermal nociception was investigated in the mouse hot-plate test. Intracerebroventricular (i.c.v.) administration of TMB-8, a blocker of Ca^{++} release from intracellular stores, produced hypernociception. By contrast, i.c.v. pretreatment with thapsigargin, a depletor of Ca^{++} intracellular stores, produced an increase of the mouse pain threshold. Furthermore, non-analgesic doses of thapsigargin prevented the hypernociception produced by TMB-8. In mice undergoing treatment with heparin, an $InsP_3$-receptor antagonist, or ryanodine, a ryanodine receptor (RyR) antagonist, a dose-dependent reduction of the pain threshold was observed. Pretreatment with D-*myo* inositol, compound which produces $InsP_3$, and 4-chloro-*m*-cresol, a RyR agonist, induced an antinociceptive effect. The heparin hypernociception was prevented by D-*myo* inositol, but not by L-*myo* inositol, used as negative control. In the same experimental conditions, the antinociception induced by D-*myo* inositol was prevented by a non-hyperalgesic dose of heparin. Similarly, the reduction of pain threshold produced by ryanodine was reversed by non-analgesic doses of 4-chloro-*m*-cresol, whereas the antinocicpetion induced by 4-chloro-*m*-cresol was prevented by non-hyperalgesic doses of ryanodine. The pharmacological treatments employed did not produce any behavioral impairment of mice as revealed by the rota-rod and hole-board tests. These results indicate that a variation of intracellular calcium contents at a supraspinal level is involved in the modulation of acute thermal nociception. In particular, the stimulation of both $InsP_3$- and Ry-receptors appears to play an important role in the induction of antinociception in mice, whereas a blockade of these receptors is involved in an hypernociceptive response to acute thermal pain.

Keywords: InsP3-receptor; Ry-receptor; Analgesia; Hyperalgesia; Inositol 1,4,5-trisphosphate; Intracellular Ca^{++}

1. Introduction

Calcium ions are widely recognized to play a fundamental role in the regulation of several biological processes. Transient changes in cytoplasmic Ca^{2+} concentration represent a key step for neurotransmitter release and the modulation of cell membrane excitability. Evidence has also accumulated for the involvement of Ca^{2+} in nociception and antinociception.

The involvement of Ca^{2+} in the transmission of nociceptive signals has been observed at the spinal cord level, suggesting a role of intracellular Ca^{2+} levels for the development of persistent pain, but not in the transmission of inputs in response to brief noxious stimuli. Selective N-type Ca^{2+} channel antagonists were antinociceptive in an animal model of neuropathic pain (Bowersox et al., 1996). The increase of intracellular Ca^{2+} by means of intrathecal (i.t.) Ca^{2+} ionophores or Ca^{2+} agonists increased the response to formalin test (Coderre and Melzack, 1992). The Ca^{2+} chelators (i.t.) reduced the second, but not the first phase of the formalin test (Coderre and Melzack, 1992). It has also been supposed that thermal allodynia and hyperalgesia in diabetic mice is related to an increase of intracellular Ca^{2+} contents (Kamei et al., 2000). Conversely, it has been observed that i.t. calcium chloride produced antinociception against acute pain (Smith and Dewey, 1992). Furthermore, the i.t. administration of drugs that increase intracellular Ca^{2+} levels enhanced nicotine-induced antinociception, whereas i.t. administration of agents that decrease intracellular Ca^{2+} blocked the nicotine increase of pain threshold (Damaj et al., 1993).

On the role of intracellular Ca^{2+} at a supraspinal levels, there are several reports indicating its involvement, sometimes opposed, in the mechanism of action of antinociceptive drugs in models of acute pain. It is

* Corresponding author. Tel.: +39-055-4271312; fax: +39-055-4271280.
E-mail address: nicoletta.galeotti@unifi.it (N. Galeotti).

0028-3908/$ - see front matter © 2004 Published by Elsevier Ltd.
doi:10.1016/j.neuropharm.2004.07.001

well documented that agents that increase cytosolic Ca^{2+} block antinociception induced by μ-opioid agonists when injected intracerebroventricularly (i.c.v.) (Smith and Stevens, 1995; Harris et al., 1975; Vocci et al., 1980; Ohsawa et al., 1998). Furthermore, Ca^{2+} chelators or antagonists of L-, N- and P-type Ca^{2+} channels, potentiate μ-opioid receptor-mediated antinociception (Ohsawa et al., 1998; Prado, 2001). Conversely, the antinociception produced by selective μ-opioid receptor agonists was potentiated by agents that increase intracellular Ca^{2+}, whereas it was reduced by i.c.v. pretreatment with EGTA (Ohsawa et al., 1998). Antinociception produced by cholinomimetic drugs is prevented by i.c.v. pretreatment with compounds that inhibit the increase of intracellular Ca^{2+} levels (Galeotti et al., 2003).

The concentration of intracellular Ca^{2+} is regulated by various mechanisms related to physiological functions. One mechanism is the influx of Ca^{2+} via Ca^{2+} channels through the plasma membrane. Another is the release of Ca^{2+} from intracellular stores via intracellular Ca^{2+}-release channels, the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) and the ryanodine receptor (RyR): $InsP_3R$ is a key molecule for $InsP_3$-induced Ca^{2+} release, whereas RyR is important for Ca^{2+}-induced Ca^{2+} release (Mikoshiba, 1997; Fill and Copello, 2002).

Even if the role of supraspinal intracellular Ca^{2+} in the mechanism of analgesic drugs has been investigated, little is known on its involvement on the physiological mechanisms of pain perception. The aim of this study was, therefore, to determine the role of $InsP_3$- and Ry-receptors in the modulation of acute thermal pain perception.

2. Methods

2.1. Animals

Male Swiss albino mice (24–26 g) from Morini (San Polo d'Enza, Italy) were used. Twelve mice were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet and tap water ad libitum and kept at 22 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m. All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory animals. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

2.2. Hot-plate test

The method adopted was described by O'Callaghan and Holtzman (1975). The mice were placed inside a stainless steel container, which was set thermostatically at 52.5 ± 0.1 °C in a precision water bath from KW Mechanical Workshop, Siena, Italy. Reaction times (s) were measured with a stopwatch before and 15, 30, 45 and 60 min after administration of hyperalgesic drugs or every 30 min up to 210 min for analgesic drugs. The endpoint used was the licking of the fore or hind paws. Those mice scoring less than 12 and more than 18 s in the pretest were rejected (30%). An arbitrary cut-off time of 45 s was adopted to avoid. No sign of tissue injury was observed up to 45 s. Fourteen mice per group were tested.

2.3. Rota-rod test

The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus, up to five mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s according to Vaught et al. (1985). Those mice scoring less than 3 and more than 6 falls in the pretest were rejected (20%). The performance time was measured before (pretest) and 15, 30 and 45 min after the beginning of the test. Ten mice per group were tested.

2.4. Hole-board test

The hole-board test consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed 4×4 in an equidistant, grid-like manner. Mice were placed on the center of the board one by one and allowed to move about freely for a period of 5 min each. Two electric eyes, crossing the plane from mid-point to mid-point of opposite sides, thus dividing the plane into four equal quadrants, automatically signaled the movement of the animal (counts in 5 min) on the surface of the plane (spontaneous motility). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice. Ten mice per group were tested.

2.5. I.c.v. injection technique

I.c.v. administration was performed under ether anesthesia with isotonic saline as solvent, according to the method described by Haley and McCormick (1957). During anesthesia, mice were grasped firmly by the loose skin behind the head. A hypodermic needle (0.4 mm external diameter) attached to a 10 μl syringe was inserted perpendicularly through the skull and no

more than 2 mm into the brain of the mouse, where 5 μl solution were then administered. The injection site was 1 mm to the right or left from the mid-point on a line drawn through to the anterior base of the ears. Injections were performed randomly into the right or left ventricle. To ascertain that solutions were administered exactly into the cerebral ventricle, some mice were injected with 5 μl of diluted 1:10 India ink and their brains were examined macroscopically after sectioning. The accuracy of the injection technique was evaluated with 95% of injections being correct.

2.6. Drugs

The following drugs were used: TMB-8 (8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate) hydrochloride, heparin sodium salt (molecular weight: approximately 60 000) (Sigma, Milan, Italy); ryanodine, 4-chloro-*m*-cresol, thapsigargin, D-*myo*-inositol 1,4,5-trisphosphate hexasodium salt, L-*myo*-inositol 1,4,5-trisphosphate hexapotassium salt (Calbiochem, Milan, Italy); D-amphetamine (De Angeli, Rome, Italy). Other chemicals were of the highest quality commercially available.

Thapsigargin was dissolved in 10% DMSO, 4-chloro-*m*-cresol (4-Cmc) was dissolved in 0.5% ethanol, whereas all other drugs were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 5 μl per mouse by i.c.v. injection and 10 ml kg^{-1} by subcutaneous (s.c.) injection.

2.7. Statistical analysis

All experimental results are given as the mean ± S.E.M. An analysis of variance, ANOVA, followed by Fisher's protected least significant difference procedure for post hoc comparison, were used to verify significance between two means of behavioral results. Data were analyzed with the StatView software for the Macintosh (1992). *P* values of less than 0.05 were considered significant.

3. Results

3.1. Effect of TMB-8 and thapsigargin on mouse pain threshold

The effect of intracellular Ca^{++} modulators on mouse pain threshold was evaluated by means of the mouse hot-plate test. TMB-8, a blocker of Ca^{++} release from intracellular stores, induced hyperalgesia in a dose-dependent manner. The doses of 0.1 and 1 μg per mouse i.c.v. were devoid of any effect, whereas at 5 μg per mouse i.c.v. TMB-8 significantly reduced the pain threshold reaching its maximum effect at 10 μg per mouse i.c.v. (Fig. 1A). Higher doses were not investigated since they induced mild side effects.

Thapsigargin, an inhibitor of Ca^{++} uptake into the endoplasmic reticulum by inhibiting the sarco-endoplasmatic reticulum Ca^{++}-ATPases, produced a dose-dependent antinociception. The increase of the pain threshold reached the statistical significance at 15 nmol per mouse i.c.v. and remained unchanged at 20 nmol per mouse i.c.v. Lower doses were devoid of any effect (Fig. 1B).

The hyperalgesic effect of TMB-8 was dose-dependently reversed by non-antinociceptive doses of thapsigargin. Thapsigargin, at 3 nmol per mouse i.c.v., was unable to modify the decrease of pain threshold produced by TMB-8 (10 μg per mouse i.c.v.), whereas at 9 nmol per mouse i.c.v., a complete reversal of the TMB-8 effect was observed (Fig. 2).

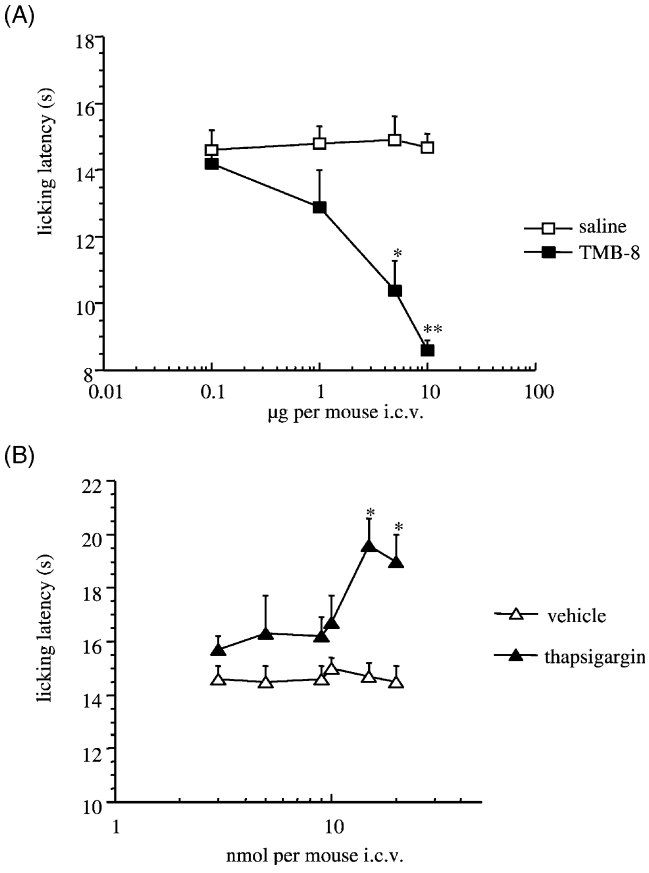

Fig. 1. Panel A: Dose–response curve of TMB-8 (0.1–10 μg per mouse i.c.v.) in the mouse hot-plate test. The licking latency value were recorded 15 min after TMB-8 administration. Vertical lines represent S.E.M. * $P < 0.05$, ** $P < 0.01$ in comparison with saline-treated group. Panel B: Dose–response curve of thapsigargin (3–20 nmol per mouse i.c.v.) in the mouse hot-plate test. The licking latency value were recorded 120 min after thapsigargin administration. Vertical lines represent S.E.M. * $P < 0.05$ in comparison with vehicle-treated group.

The licking latency values reported in Figs. 1 and 2 were recorded in correspondence with TMB-8 and thapsigargin maximum effect (15 and 120 min after administration, respectively) as evidenced by time-course experiments performed in our laboratory (data not shown).

3.2. Effect of IP_3R modulators on mouse pain threshold

The administration of heparin (1–40 μg per mouse i.c.v.), an antagonist of InsP3 receptors, produced a dose-dependent decrease of the pain threshold, reaching its maximum effect at 80 μg per mouse i.c.v. (Fig. 3A). The licking latency values reported in the figure were recorded 15 min after administration of heparin in correspondence with its maximum effect as evidenced by time-course experiments (data not shown). Higher doses were not investigated since evident signs of toxicity appeared. D-*myo* inositol, at the dose of 10 μg per mouse i.c.v., produced antinociception which reached the statistical significance 90 min after administration, peaked after 120 min and then it slowly diminished completely disappearing after 210 min. The dose of 1 μg per mouse i.c.v. was devoid of any effect (Fig. 3B).

The hyperalgesia produced by heparin (80 μg per mouse i.c.v.) was reversed by pretreatment with D-*myo* inositol in a dose-dependent manner. The dose of D-*myo* inositol of 0.01 μg per mouse i.c.v. was completely ineffective, the dose of 0.1 μg per mouse i.c.v. produced a partial reversal without reaching the statistical significance, whereas the dose of 1 and 5 μg per mouse i.c.v.

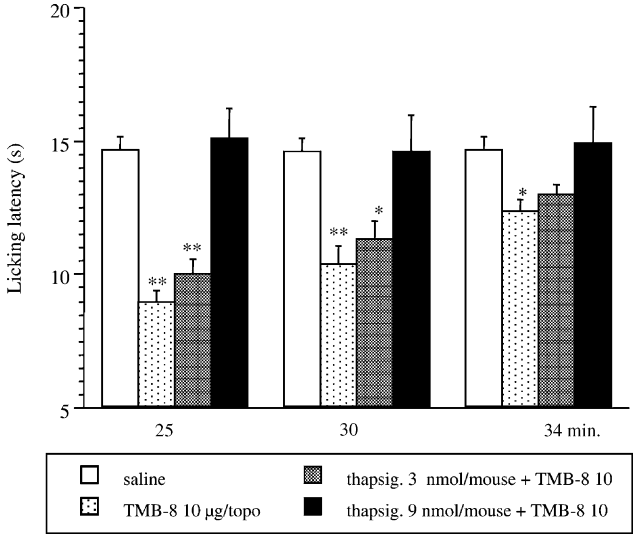

Fig. 2. Prevention by pretreatment with thapsigargin (3–9 nmol per mouse i.c.v.) of TMB-8 (10 μg per mouse i.c.v.)-induced hyperalgesia in the mouse hot-plate test. The licking latency values were recorded 15 min after TMB-8 administration. Thapsigargin was injected 105 min before administering TMB-8. Vertical lines represent S.E.M. * $P < 0.05$, ** $P < 0.01$ in comparison with saline-treated mice.

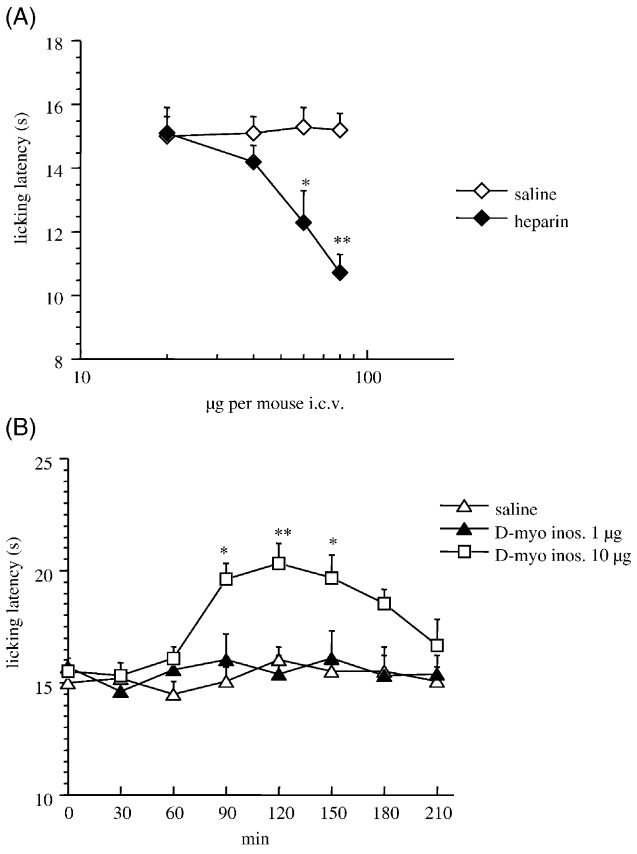

Fig. 3. Panel A: Dose–response curve of heparin (20–80 μg per mouse i.c.v.) in the mouse hot-plate test. The licking latency value were recorded 15 min after heparin administration. Vertical lines represent S.E.M. * $P < 0.05$, ** $P < 0.01$ in comparison with saline-treated group. Panel B: Dose–response curve of D-*myo* inositol (1–10 μg per mouse i.c.v.) in the mouse hot-plate test. Vertical lines represent S.E.M. * $P < 0.05$ in comparison with saline-treated group.

produced the antagonism of the heparin-induced hyperalgesic effect. The administration of L-*myo* inositol, used as negative control, did not modify the hyperalgesia induced by heparin (Fig. 4). Furthermore, the antinociceptive effect produced by D-*myo* inositol (10 μg per mouse i.c.v.) was antagonized by administration of heparin at 40 μg per mouse i.c.v. (Fig. 4).

3.3. Effect of RyR modulators on mouse pain threshold

The i.c.v. administration of ryanodine, a selective antagonist of RyR, dose-dependently reduced the licking latency values in the mouse hot-plate test. Ryanodine, at 0.03 nmol per mouse, was devoid of any effect. The dose of ryanodine of 0.06 nmol per mouse reduced the licking latency values without reaching the statistical significance whereas the doses of 0.1–3 nmol per mouse significantly reduced the pain threshold inducing an hyperalgesic effect. The maximum effect was reached at 1 nmol per mouse i.c.v. (Fig. 5A).

The administration of 4-Cmc, an agonist of RyR, produced an increase of the pain threshold in a dose-

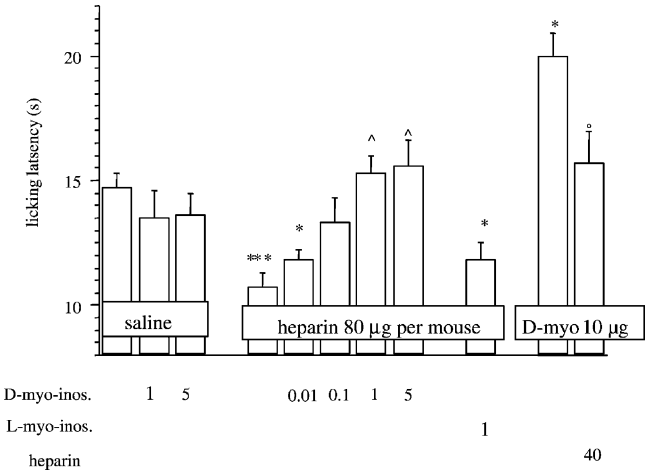

Fig. 4. Reversal by pretreatment with D-*myo* inositol (0.01–5 µg per mouse i.c.v.) of heparin (80 µg per mouse i.c.v.)-induced hyperalgesia and prevention by heparin (40 µg per mouse i.c.v.) of D-*myo* inositol (10 µg per mouse i.c.v.)-induced antinociception in the mouse hot-plate test. The licking latency values were recorded 15 min after heparin administration. D-*myo* inositol was injected 105 min before administering heparin. Vertical lines represent S.E.M. * $P < 0.05$, *** $P < 0.001$ in comparison with saline-treated mice; ^ $P < 0.05$ in comparison with heparin (80 µg per mouse i.c.v.); ° $P < 0.05$ in comparison with D-*myo* inositol (10 µg per mouse i.c.v).

dependent manner, reaching its maximum effect at 1 nmol per mouse i.c.v. since at 3 nmol per mouse, the licking latency values were not further increased (Fig. 5B).

The hyperalgesia induced by ryanodine at the doses of 0.1 and 1 nmol per mouse i.c.v. was dose-dependently reversed by 4-Cmc (0.003–0.3 nmol per mouse i.c.v.) (Fig. 6). Furthermore, the antinociception induced by 4-Cmc (1 nmol per mouse i.c.v.) was prevented by ryanodine, i.c.v. administered at the dose of 0.03 nmol per mouse (Fig. 6). The i.c.v. injection of 4-Cmc (0.3 nmol per mouse i.c.v.) and ryanodine (0.03 nmol per mouse i.c.v.) alone did not modify the licking latency of mice in comparison with saline-treated animals (Fig. 6).

The licking latency values reported in Figs. 5 and 6 were recorded in correspondence with ryanodine and 4-Cmc maximum effect (15 and 120 min after administration, respectively) as evidenced by time-course experiments performed in our laboratory (data not shown).

3.4. Effect of treatments on mouse behavior

The compounds investigated, at the highest effective doses, were tested in order to assess their effect on mouse behavior. Mice pretreated with TMB-8 (10 µg per mouse i.c.v.), heparin (80 µg per mouse i.c.v.), ryanodine (1 nmol per mouse i.c.v.), 4-Cmc (1 nmol per mouse i.c.v.), thapsigargin (20 nmol per mouse i.c.v.) and D-*myo* inositol (10 µg per mouse i.c.v.) were evaluated for motor coordination by use of the rota-rod test, and for spontaneous motility and inspection activity by use of the hole-board test.

The number of falls from the rotating rod, evaluated before and 15, 30 and 45 min after the beginning of the rota-rod test, showed the lack of any impairment in the motor coordination of animals pretreated with all pharmacological modulators in comparison with the control group (Fig. 7).

The spontaneous motility as well as the inspection activity of mice, expressed as counts in 10 min, were unmodified by pretreatment with TMB-8, heparin, ryanodine, 4-Cmc, thapsigargin and D-*myo* inositol in comparison with control group (Fig. 8).

4. Discussion

The present study investigated the role of intracellular Ca^{2+} in the supraspinal pain perception in a condition of acute thermal nociception in mice. Ca^{2+} plays an important role in a variety of central and peripheral physiological processes. To avoid the possible appearance of peripheral effects that could lead to a misinterpretation of the results obtained, the Ca^{2+} modulators used in the present study were administered directly into the cerebral ventricles.

The i.c.v. administration of TMB-8, an agent that antagonizes the mobilization of Ca^{++} from intracellular stores (Malagodi and Chiou, 1974), produced a dose-dependent reduction of the mouse pain threshold. A previous study reported that TMB-8, administered i.c.v., produced antinociception in the tail flick test (Welch and Dewey, 1986). However, it should be noted that the analgesic effect of TMB-8 was obtained with doses (ED_{50}: 50 µg per mouse) about 10 times higher than those used in the present study. Thus, it seems likely that low doses of TMB-8 enhance the nociceptive response, while relatively higher doses of TMB-8 produce antinociception in mice. This hypothesis cannot be verified since, in our experimental conditions, doses of TMB-8 higher than 10 µg per mouse induced behavioral side effects.

By contrast, the i.c.v. administration of thapsigargin, compound which selectively inhibits Ca^{2+} uptake into the endoplasmic reticulum by inhibiting the sarco-endoplasmic reticulum ATPases (SERCAs) and thus increasing the intracellular Ca^{2+} concentration (Treiman et al., 1998), induced an antinociceptive effect. These results are in agreement with previous data reporting an increase of the pain threshold produced by i.t. administration of thapsigargin in the mouse tail flick test (Bernstein and Welch, 1995; Alvarez-Vega et al., 2001). Furthermore, pretreatment with non-analgesic doses of thapsigargin, a prevention of the TMB-8 induced hyperalgesia was obtained confirming

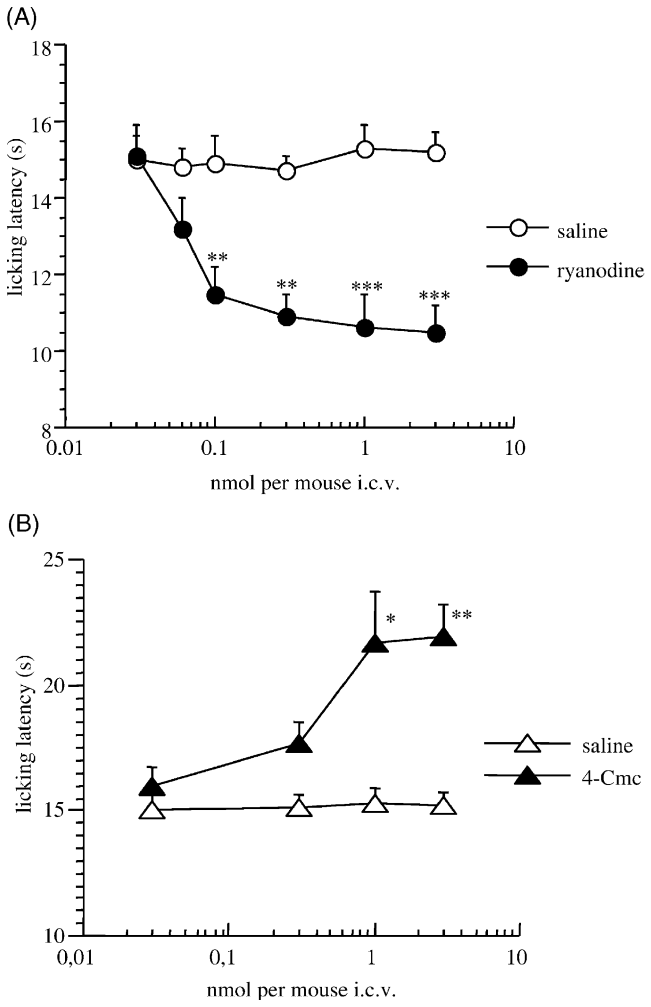

Fig. 5. Panel A: Dose–response curve of ryanodine (0.03–3 nmol per mouse i.c.v.) in the mouse hot-plate test. The licking latency value were recorded 15 min after ryanodine administration. Vertical lines represent S.E.M. ** $P < 0.01$, *** $P < 0.001$ in comparison with saline-treated group. Panel B: Dose–response curve of 4-Cmc (0.03–3 nmol per mouse i.c.v.) in the mouse hot-plate test. The licking latency value were recorded 120 min after 4-Cmc administration. Vertical lines represent S.E.M. * $P < 0.05$, ** $P < 0.01$ in comparison with saline-treated group.

that the hyperalgesia produced by TMB-8 was related to a reduction of the intracellular Ca^{2+} levels. These observations suggest that a supraspinal reduction of intracellular calcium contents induces hypersensitivity to an acute thermal stimulus.

The release of Ca^{2+} from intracellular stores is mediated via intracellular Ca^{2+}-release channels, the $InsP_3R$ and the RyR: $InsP_3R$ is a key molecule for $InsP_3$-induced Ca^{2+} release, whereas RyR is important for Ca^{2+}-induced Ca^{2+} release. The involvement of these two receptor subtypes in the modulation of pain perception was, therefore, investigated. The i.c.v. administration of heparin, a potent $InsP_3$-receptor antagonist (Jonas et al., 1997), produced a dose-dependent hyperalgesic effect indicating the importance of $InsP_3R$ in the modulation of pain perception. This hypothesis is supported by data indicating that the blockade of $InsP_3R$ by heparin, as well as the inhibition of $InsP_3$ production by LiCl, prevented the analgesic effect of cholinomimetic drugs (Galeotti et al., 2003) and delta opioid agonists (Narita et al., 2000). Heparin must be injected into cells or perfused onto permeabilized cells because of its high molecular weight (12 000–13 000 Da) and lack of membrane permeability. Some evidence indicates that the low molecular weight heparin (6000 Da) used in this study is membrane permeable. Perfusion of low molecular weight heparin over a non-permeabilized cerebellar slice preparation attenuated glutamate-stimulated increases in free intracellular Ca^{++} (Jonas et al., 1997). Conversely, the i.c.v injection of D-*myo* inositol, compound which generates $InsP_3$, produced an increase of the mouse pain threshold that was prevented by non-hyperalgesic doses of heparin. Heparin may act through other cellular mechanisms. Heparin is also a potent inhibitor of G protein-coupled receptor kinases that regulate the responsiveness of receptors (i.e. opioid receptors) involved in the modulation of pain perception (Kuna-

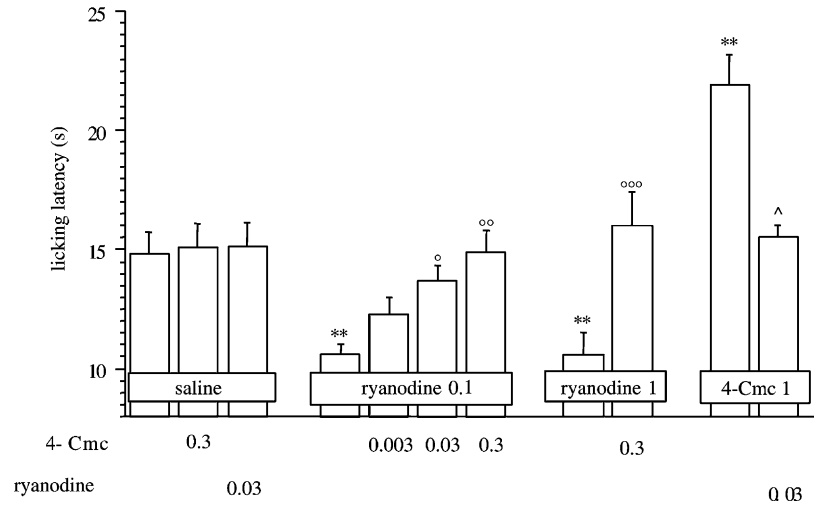

Fig. 6. Reversal by pretreatment with 4-Cmc (0.003–0.3 nmol per mouse i.c.v.) of ryanodine (0.1–1 nmol per mouse i.c.v.)-induced hyperalgesia and prevention by ryanodine (0.03 nmol per mouse i.c.v.) of 4-Cmc (1 nmol per mouse i.c.v.)-induced antinociception in the mouse hot-plate test. The licking latency values were recorded 15 min after ryanodine administration. 4-Cmc was injected 105 min before administering ryanodine. Vertical lines represent S.E.M. ** $P < 0.01$ in comparison with saline-treated mice; $^{\circ}$ $P < 0.05$, $^{\circ\circ}$ $P < 0.01$ in comparison with ryanodine (0.1 nmol per mouse i.c.v.), $^{\circ\circ\circ}$ $P < 0.05$ in comparison with ryanodine (1 nmol per mouse i.c.v.); $^{\wedge}P < 0.05$ in comparison with 4-Cmc (1 nmol per mouse i.c.v).

puli et al., 1994). The decrease of pain threshold produced by heparin was dose-dependently reversed by D-*myo* inositol, but not by L-*myo* inositol, used as negative control, confirming that the heparin effect observed in the present study was due to the blockade of $InsP_3$ receptors.

The i.c.v. administration of ryanodine produced a dose-dependent hyperalgesic effect in mice. It has been

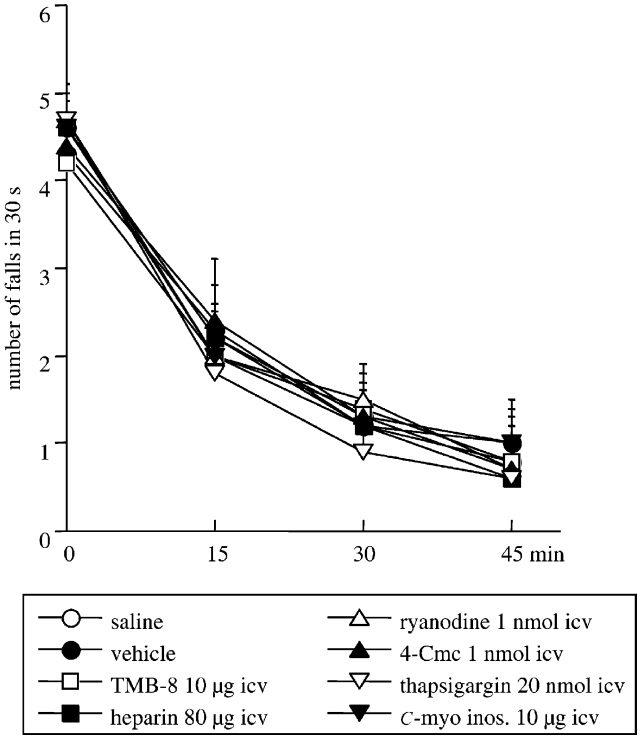

Fig. 7. Lack of effect by TMB-8 (10 µg per mouse i.c.v.), heparin (80 µg per mouse i.c.v.), ryanodine (1 nmol per mouse i.c.v.), 4-Cmc C (1 nmol per mouse i.c.v.), thapsigargin (20 nmol per mouse i.c.v.) and D-*myo* inositol (10 µg per mouse i.c.v.) on motor coordination in the mouse rota-rod test. Vertical lines represent S.E.M. 4-Cmc, thapsigargin and D-*myo* inositol were injected 105 min before the beginning of the test, whereas TMB-8, heparin and ryanodine were administered 15 min before the beginning of the test. Vehicle: 10% DMSO.

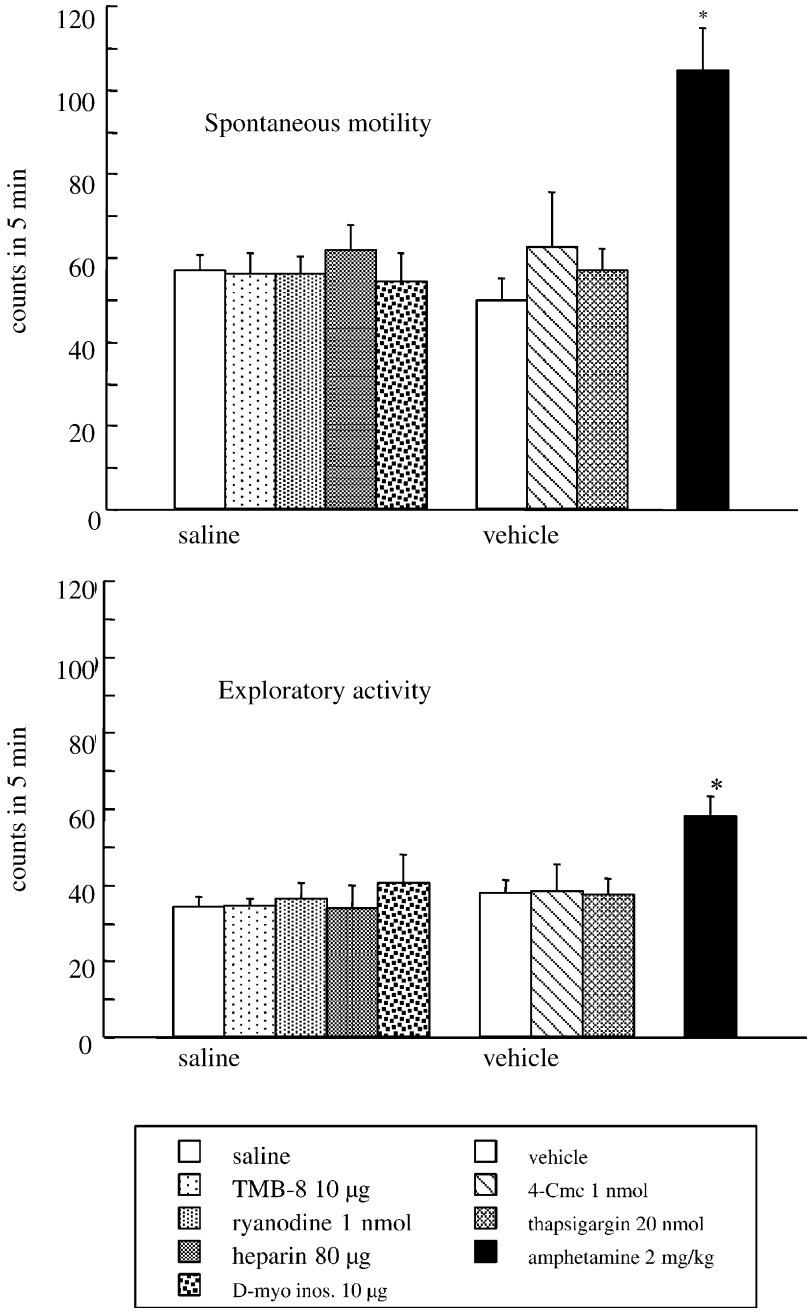

Fig. 8. Lack of effect by TMB-8 (10 µg per mouse i.c.v.), heparin (80 µg per mouse i.c.v.), ryanodine (1 nmol per mouse i.c.v.), 4-Cmc C (1 nmol per mouse i.c.v.), thapsigargin (20 nmol per mouse i.c.v.) and D-*myo* inositol (10 µg per mouse i.c.v.) on spontaneous motility and exploratory activity in the mouse hole-board test in comparison with saline or vehicle. Vertical lines represent S.E.M. 4-Cmc, thapsigargin and D-*myo* inositol were injected 120 min before the beginning of the test, whereas TMB-8, heparin and ryanodine were administered at the beginning of the test. Vehicle: 10% DMSO.

reported that ryanodine blocks Ca^{2+} release from Ca^{2+}/caffeine-sensitive microsomal pools, which are involved in the phenomenon of Ca^{2+}-induced Ca^{2+} release (McPherson et al., 1991). Ryanodine reduces the rate at which $[Ca^{2+}]_i$ increase with Ca^{2+} entry (Friel and Tsien, 1992). Thus, it seems likely the nociceptive response produced by i.c.v. ryanodine may be due to a decrease of $[Ca^{2+}]_i$ at supraspinal level. It has been reported that intrathecal injection of ryanodine did not produce any effect on the mouse pain threshold in the tail flick and formalin tests (Ohsawa and Kamei, 1999; Kamei et al., 2000). On these bases, we can hypothesise that variations of cytosolic Ca^{2+} contents induced by RyR differentially modulates the pain perception at spinal and supraspinal level. To confirm this hypothesis, the effect of 4-chloro-*m*-cresol

(4-Cmc), an agonist of RyR (Herrmann-Frank and Varsanyi, 1993), was tested. 4-Cmc induced an increase of the mouse pain threshold after i.c.v. administration that was prevented by non-hyperalgesic doses of ryanodine. Furthermore, the ryanodine-mediated hyperalgesia was prevented by non-analgesic doses of 4-Cmc, excluding the possibility that the ryanodine effect was due to the induction of a non-specific action not mediated by RyR blockade. It should also be taken into account that compounds able to activate RyR, such as caffeine, are endowed with central antinociceptive properties (Person et al., 1985; Sawynok et al., 1995; Ghelardini et al., 1997).

Cytosolic Ca^{2+} regulates numerous neuronal functions (Berridge, 1998) and, therefore, a variation of intracellular Ca^{2+} contents can induce behavioral side effects. All the Ca^{2+} modulators used in the present study, at the highest active doses employed, did not cause any detectable modification in mouse gross behavior. At the same doses, all treatments did not impair motor coordination nor modify spontaneous motility nor inspection activity in comparison with control groups excluding that the results obtained were due to animals' altered viability.

Seen as a whole, present results indicate that the variation of cytosolic Ca^{2+} contents is involved in the modulation of acute thermal pain perception at the supraspinal level. In particular, an increase of intracellular Ca^{2+} enhances the pain threshold whereas a decrease of cytosolic Ca^{2+} renders animal hypersensitive to a thermal painful stimulus. Furthermore, both $InsP_3R$ and RyR appear to participate to the modulation of acute thermal pain perception.

Acknowledgements

This work was supported by the grants from MIUR.

References

Alvarez-Vega, M., Baamonde, A., Hidalgo, A., Menendez, L., 2001. Effects of the calcium release inhibitor dantrolene and the Ca^{2+}-ATPase inhibitor thapsigargin on spinal nociception in rats. Pharmacology 62 (3), 145–150.

Bernstein, M.A., Welch, S.P., 1995. Alteration in L-type calcium channels in the brain and spinal cord of acutely treated and morphine-tolerant mice. Brain Research 696, 83–88.

Berridge, M.J., 1998. Neuronal calcium signaling. Neuron 21, 13–26.

Bowersox, S.S., Gadbois, T., Singh, T., Pettus, M., Wang, Y.-X., Luther, R.R., 1996. Selective N-type neuronal voltage-sensitive calcium channel blockers, SNX-111, produces spinal antinociception in rat model of acute, persistent and neuropathic pain. Journal of Pharmacology and Experimental Therapeutics 279, 1243–1249.

Coderre, T.J., Melzack, R., 1992. The role of NMDA receptor-operated calcium channels in persistent nociception after formalin-induced tissue injury. Journal of Neuroscience 12, 3671–3675.

Damaj, M.I., Welch, S.P., Martin, B.R., 1993. Involvement of calcium and L-type channels in nicotine-induced antinociception. Journal of Pharmacology and Experimental Therapeutics 266, 1330–1338.

Fill, M., Copello, J.A., 2002. Ryanodine receptor calcium release channels. Physiological Reviews 82, 893–922.

Friel, D.D., Tsien, R.W., 1992. A caffeine- and ryanodine-sensitive Ca^{2+} store in bullfrog sympathetic neurons modulates effects of Ca^{2+} entry on [Ca^{2+}]. Journal of Physiology 450, 217–246.

Galeotti, N., Bartolini, A., Ghelardini, C., 2003. The phospholipase C-IP_3 pathway is involved in muscarinic antinociception. Neuropsychopharmacology 28, 888–897.

Ghelardini, C., Galeotti, N., Bartolini, A., 1997. Caffeine induces central cholinergic analgesia. Naunyn-Schmiedebergs Archives of Pharmacology 356, 590–595.

Haley, T.J., McCormick, W.G., 1957. Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. British Journal of Pharmacology and Chemotherapy 12, 12–15.

Harris, R.A., Loh, H.H., Way, E.L., 1975. Effects of divalent cations, cation chelators and an ionophore on morphine analgesia and tolerance. Journal of Pharmacology and Experimental Therapeutics 195, 488–498.

Herrmann-Frank, A., Varsanyi, M., 1993. Enhancement of Ca^{2+} release channel activity by phosphorylation of the skeletal muscle ryanodine receptor. FEBS Letters 332, 237–242.

Jonas, S., Sugimori, M., Llinas, R., 1997. Is low molecular weight heparin a neuroprotectant? Annals of the New York Academy of Science 825, 389–393.

Kamei, J., Taki, K., Ohsawa, M., Hitosugi, H., 2000. Modulation of the formalin-induced nociceptive response by diabetes: possible involvement of intracellular calcium. Brain Research 862, 257–261.

Kunapuli, P., Onorato, J.J., Hosey, M.M., Benovic, J.L., 1994. Expression, purification, and characterization of the G protein-coupled receptor kinase GRK5. Journal of Biological Chemistry 269, 1099–1105.

Malagodi, M.H., Chiou, C.Y., 1974. Pharmacological evaluation of a new Ca^{2+} antagonist, 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8): studies in smooth muscles. European Journal of Pharmacology 27, 25–33.

McPherson, P.S., Kim, Y.K., Valdivia, H., Knudson, C.M., Takekura, H., Franzini-Armstrong, C., Coronado, R., Campbell, K.P., 1991. The brain ryanodine receptor: a caffeine-sensitive calcium release channel. Neuron 7, 17–25.

Mikoshiba, K., 1997. The InsP3 receptor and intracellular Ca^{2+} signaling. Current Opinion in Neurobiology 7, 339–345.

Narita, M., Ohsawa, M., Mizoguchi, H., Aoki, T., Suzuki, T., Tseng, L.F., 2000. Role of the phosphatidylinositol-specific phospholipase C pathway in delta-opioid receptor-mediated antinociception in the mouse spinal cord. Neuroscience 99, 327–331.

O'Callaghan, J.P., Holtzman, S.G., 1975. Quantification of the analgesic activity of narcotic antagonists by a modified hot-plate procedure. Journal of Pharmacology and Experimental Therapeutics 192, 497–505.

Ohsawa, M., Kamei, J., 1999. Role of intracellular calcium in thermal allodynia and hyperalgesia in diabetic mice. Brain Research 833, 278–281.

Ohsawa, M., Nagase, H., Kamei, J., 1998. Role of intracellular calcium in modification of mu and delta opioid receptor-mediated antinociception by diabetes in mice. Journal of Pharmacology and Experimental Therapeutics 286, 780–787.

Person, D.L., Kissin, I., Brown, P.T., Xavier, A.V., Vinik, H.R., Bradley, E.L., 1985. Morphine–caffeine analgesic interaction in rats. Anesthesia and Analgesia 64, 851–856.

Prado, W.A., 2001. Involvement of calcium in pain and antinociception. Brazilian Journal of Medical and Biological Research 34, 449–461.

Sawynok, J., Reid, A.R., Doak, G.J., 1995. Caffeine antinociception in the rat hot-plate and formaline tests and locomotor stimulation: involvement of noradrenergic mechanisms. Pain 61, 203–207.

Smith, F.L., Dewey, W.L., 1992. Evidence that endogenous opioids mediate the antinociceptive effects of intrathecally administered calcium in mice. Pain 85, 9–18.

Smith, F.L., Stevens, D.L., 1995. Calcium modulation of morphine analgesia: role of calcium channels and intracellular pool calcium. Journal of Pharmacology and Experimental Therapeutics 272, 290–299.

Treiman, M., Caspersen, C., Christensen, S.B., 1998. A tool coming of age: thapsigargin as an inhibitor of sarco-endoplasmic reticulum Ca(2+)-ATPases. Trends in Pharmacological Sciences 19, 131–135.

Vaught, J., Pelley, K., Costa, L.G., Sether, P., Enna, S.J., 1985. A comparison of the antinociceptive responses to GABA-receptor agonists THIP and baclofen. Neuropharmacology 24, 211–216.

Vocci, Jr.., F.J., Welch, S.P., Dewey, W.L., 1980. Differential effects of divalent cations, cation chelators and an ionophore (A23187) on morphine and dibutyryl guanosine 3′: 5′-cyclic monophosphate antinociception. Journal of Pharmacology and Experimental Therapeutics 214, 463–466.

Welch, S.P., Dewey, W.L., 1986. A characterization of the antinociception produced by intracerebroventricular injection of 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate in mice. Journal of Pharmacology and Experimental Therapeutics 239, 320–326.