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# Role of intracellular calcium in acute thermal pain perception

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## 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  $\text{Ca}^{++}$  release from intracellular stores, produced hypernociception. By contrast, i.c.v. pretreatment with thapsigargin, a depletor of  $\text{Ca}^{++}$  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  $\text{InsP}_3$ -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  $\text{InsP}_3$ , 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 antinociception 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  $\text{InsP}_3$ - 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.

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**Keywords:**  $\text{InsP}_3$ -receptor; Ry-receptor; Analgesia; Hyperalgesia; Inositol 1,4,5-trisphosphate; Intracellular  $\text{Ca}^{++}$

## 1. Introduction

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

The involvement of  $\text{Ca}^{2+}$  in the transmission of nociceptive signals has been observed at the spinal cord level, suggesting a role of intracellular  $\text{Ca}^{2+}$  levels for the development of persistent pain, but not in the transmission of inputs in response to brief noxious stimuli. Selective N-type  $\text{Ca}^{2+}$  channel antagonists were antinociceptive in an animal model of neuropathic pain

(Bowersox et al., 1996). The increase of intracellular  $\text{Ca}^{2+}$  by means of intrathecal (i.t.)  $\text{Ca}^{2+}$  ionophores or  $\text{Ca}^{2+}$  agonists increased the response to formalin test (Coderre and Melzack, 1992). The  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  levels enhanced nicotine-induced antinociception, whereas i.t. administration of agents that decrease intracellular  $\text{Ca}^{2+}$  blocked the nicotine increase of pain threshold (Damaj et al., 1993).

On the role of intracellular  $\text{Ca}^{2+}$  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

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well documented that agents that increase cytosolic  $\text{Ca}^{2+}$  block antinociception induced by  $\mu$ -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,  $\text{Ca}^{2+}$  chelators or antagonists of L-, N- and P-type  $\text{Ca}^{2+}$  channels, potentiate  $\mu$ -opioid receptor-mediated antinociception (Ohsawa et al., 1998; Prado, 2001). Conversely, the antinociception produced by selective  $\mu$ -opioid receptor agonists was potentiated by agents that increase intracellular  $\text{Ca}^{2+}$ , 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  $\text{Ca}^{2+}$  levels (Galeotti et al., 2003).

The concentration of intracellular  $\text{Ca}^{2+}$  is regulated by various mechanisms related to physiological functions. One mechanism is the influx of  $\text{Ca}^{2+}$  via  $\text{Ca}^{2+}$  channels through the plasma membrane. Another is the release of  $\text{Ca}^{2+}$  from intracellular stores via intracellular  $\text{Ca}^{2+}$ -release channels, the inositol 1,4,5-trisphosphate receptor ( $\text{InsP}_3\text{R}$ ) and the ryanodine receptor ( $\text{RyR}$ ):  $\text{InsP}_3\text{R}$  is a key molecule for  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  release, whereas  $\text{RyR}$  is important for  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (Mikoshiha, 1997; Fill and Copello, 2002).

Even if the role of supraspinal intracellular  $\text{Ca}^{2+}$  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  $\text{InsP}_3$ - and  $\text{Ry}$ -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  $22 \pm 1^\circ\text{C}$  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  $52.5 \pm 0.1^\circ\text{C}$  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  $4 \times 4$  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  $\mu\text{l}$  syringe was inserted perpendicularly through the skull and no

more than 2 mm into the brain of the mouse, where 5  $\mu$ l 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  $\mu$ l 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  $\mu$ l per mouse by i.c.v. injection and 10 ml  $\text{kg}^{-1}$  by subcutaneous (s.c.) injection.

## 2.7. Statistical analysis

All experimental results are given as the mean  $\pm$  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  $\text{Ca}^{++}$  modulators on mouse pain threshold was evaluated by means of the mouse hot-plate test. TMB-8, a blocker of  $\text{Ca}^{++}$  release from intracellular stores, induced hyperalgesia in a dose-dependent manner. The doses of 0.1 and 1  $\mu$ g per mouse i.c.v. were devoid of any effect, whereas at 5

$\mu$ g per mouse i.c.v. TMB-8 significantly reduced the pain threshold reaching its maximum effect at 10  $\mu$ g per mouse i.c.v. (Fig. 1A). Higher doses were not investigated since they induced mild side effects.

Thapsigargin, an inhibitor of  $\text{Ca}^{++}$  uptake into the endoplasmic reticulum by inhibiting the sarco-endoplasmic reticulum  $\text{Ca}^{++}$ -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  $\mu$ g 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).

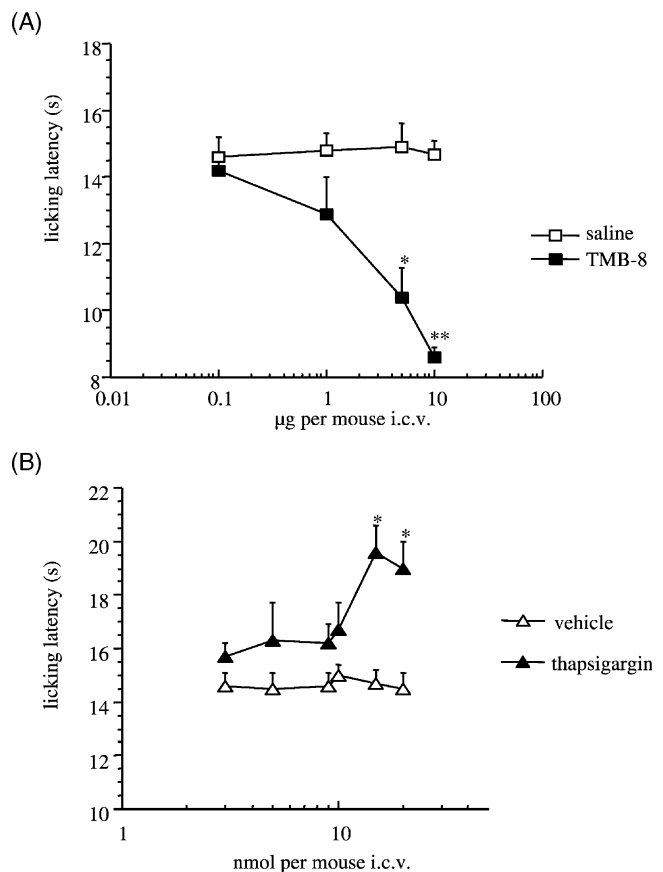


Fig. 1. Panel A: Dose-response curve of TMB-8 (0.1–10  $\mu$ g per mouse i.c.v.) in the mouse hot-plate test. The licking latency value were recorded 15 min after TMB-8 administration. Vertical lines represent S.E.M. \* *P* < 0.05, \*\* *P* < 0.01 in comparison with saline-treated group. Panel B: Dose-response 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  $\mu$ g per mouse i.c.v.), an antagonist of  $InsP_3$  receptors, produced a dose-dependent decrease of the pain threshold, reaching its maximum effect at 80  $\mu$ g 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  $\mu$ g 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  $\mu$ g per mouse i.c.v. was devoid of any effect (Fig. 3B).

The hyperalgesia produced by heparin (80  $\mu$ g 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  $\mu$ g per mouse i.c.v. was completely ineffective, the dose of 0.1  $\mu$ g per mouse i.c.v. produced a partial reversal without reaching the statistical significance, whereas the dose of 1 and 5  $\mu$ g per mouse i.c.v.

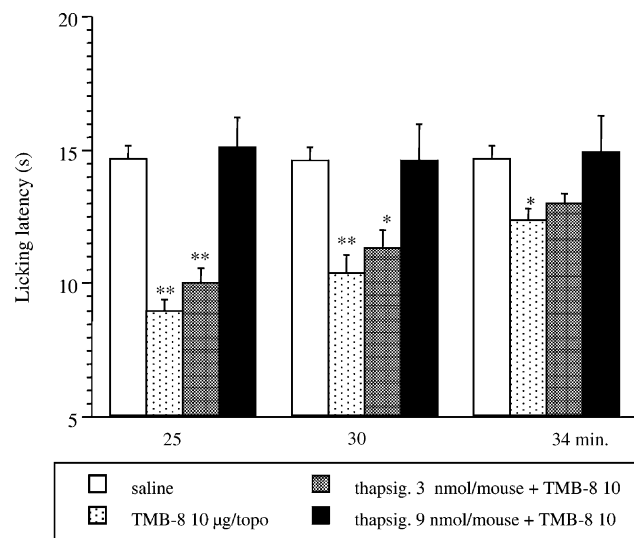


Fig. 2. Prevention by pretreatment with thapsigargin (3–9 nmol per mouse i.c.v.) of TMB-8 (10  $\mu$ g per mouse i.c.v.)-induced hyperalgesia in the mouse hot-plate test. The licking latency values were recorded 15 min after TMB-8 administration. Thapsigargin was injected 105 min before administering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

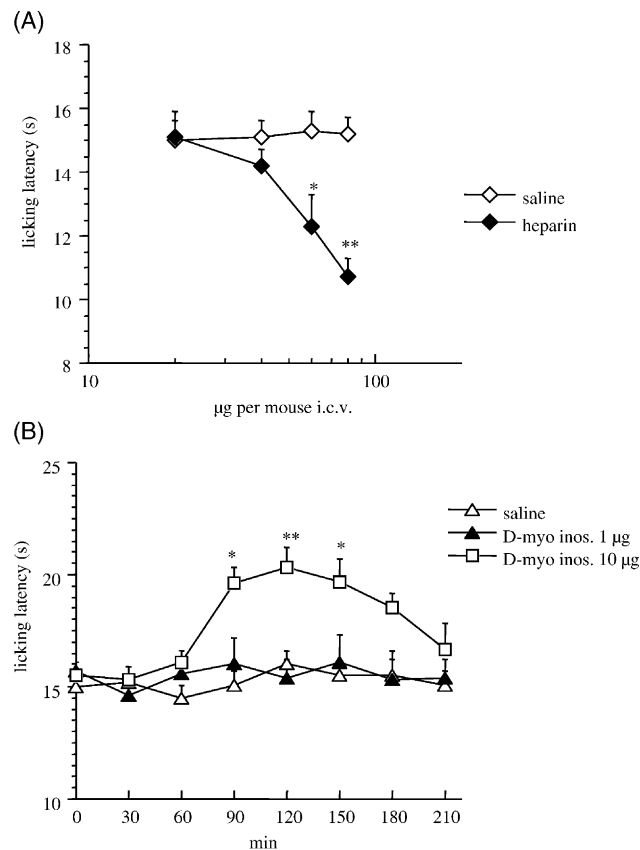


Fig. 3. Panel A: Dose-response curve of heparin (20–80  $\mu$ g per mouse i.c.v.) in the mouse hot-plate test. The licking latency value were recorded 15 min after heparin administration. Vertical lines represent S.E.M. \*  $P < 0.05$ , \*\*  $P < 0.01$  in comparison with saline-treated group. Panel B: Dose-response curve of D-myo inositol (1–10  $\mu$ g 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  $\mu$ g per mouse i.c.v.) was antagonized by administration of heparin at 40  $\mu$ g 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-



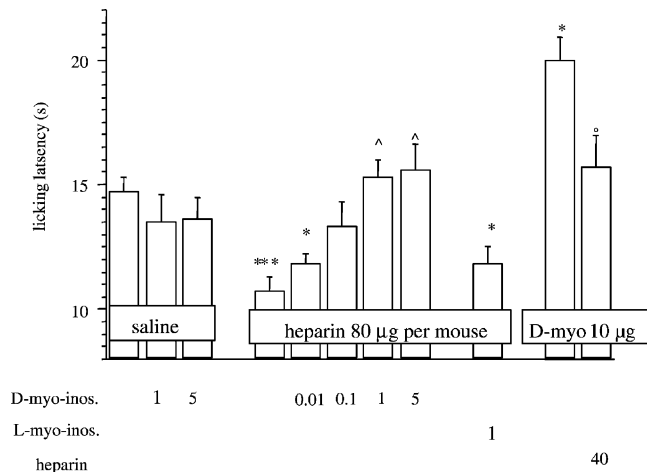


Fig. 4. Reversal by pretreatment with *D-myo* inositol (0.01–5 µg per mouse i.c.v.) of heparin (80 µg per mouse i.c.v.)-induced hyperalgesia and prevention by heparin (40 µg per mouse i.c.v.) of *D-myo* inositol (10 µg per mouse i.c.v.)-induced antinociception in the mouse hot-plate test. The licking 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 eval-

uated 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  $Ca^{2+}$  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 sarcoplasmic 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

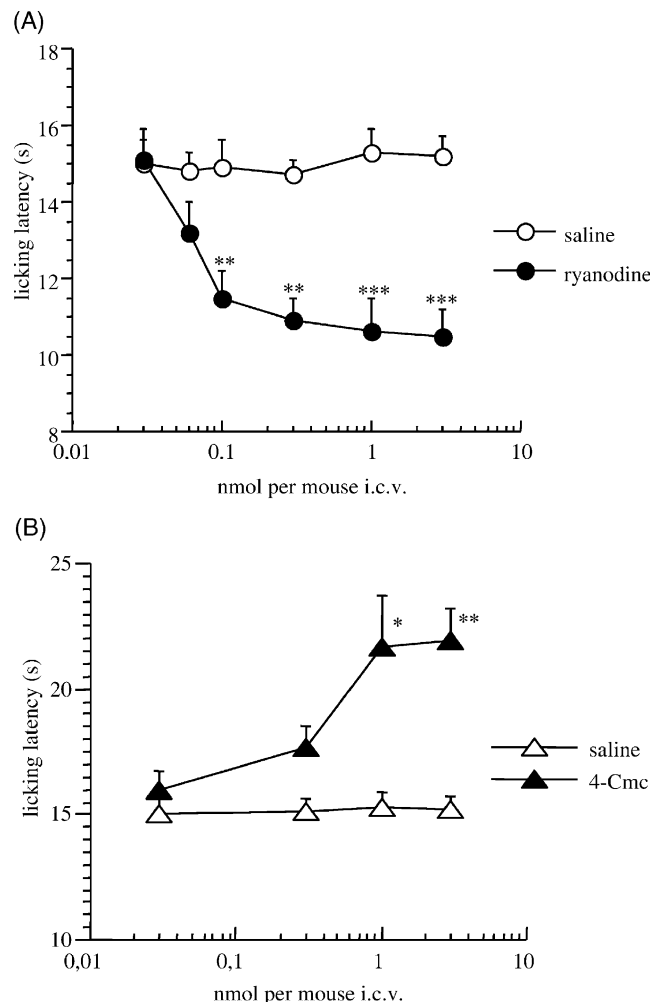


Fig. 5. Panel A: Dose–response curve of ryanodine (0.03–3 nmol per mouse i.c.v.) in the mouse hot-plate test. The licking latency value were recorded 15 min after ryanodine administration. Vertical lines represent S.E.M. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  in comparison with saline-treated group. 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  $\text{Ca}^{2+}$  levels. These observations suggest that a supraspinal reduction of intracellular calcium contents induces hypersensitivity to an acute thermal stimulus.

The release of  $\text{Ca}^{2+}$  from intracellular stores is mediated via intracellular  $\text{Ca}^{2+}$ -release channels, the  $\text{InsP}_3\text{R}$  and the  $\text{RyR}$ :  $\text{InsP}_3\text{R}$  is a key molecule for  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  release, whereas  $\text{RyR}$  is important for  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  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  $\text{InsP}_3$ -receptor antagonist (Jonas et al., 1997), produced a dose-dependent hyperalgesic effect indicating the importance of  $\text{InsP}_3\text{R}$  in the modulation of pain perception. This hypothesis is supported by data indicating that the blockade of  $\text{InsP}_3\text{R}$  by heparin, as well as the inhibition of  $\text{InsP}_3$  production by  $\text{LiCl}$ , prevented the analgesic effect of choli-

nomimetic 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  $\text{Ca}^{++}$  (Jonas et al., 1997). Conversely, the i.c.v. injection of *D-myo* inositol, compound which generates  $\text{InsP}_3$ , 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-

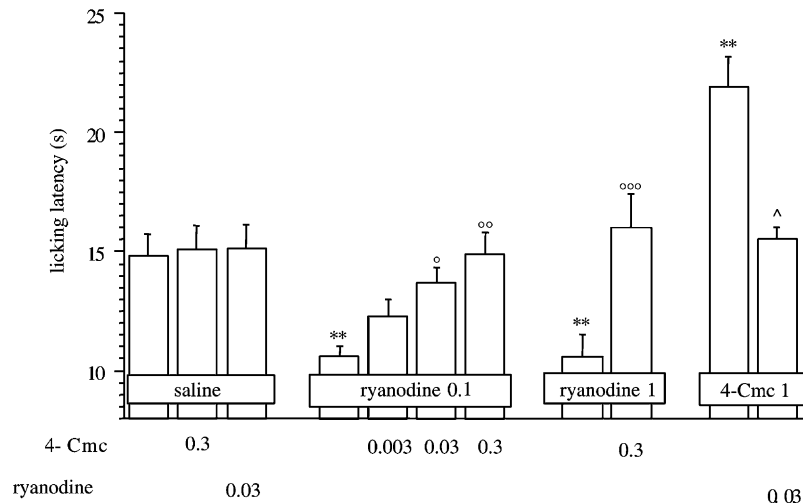


Fig. 6. Reversal by pretreatment with 4-Cmc (0.003–0.3 nmol per mouse i.c.v.) of ryanodine (0.1–1 nmol per mouse i.c.v.)-induced hyperalgesia and prevention by ryanodine (0.03 nmol per mouse i.c.v.) of 4-Cmc (1 nmol per mouse i.c.v.)-induced antinociception in the mouse hot-plate test. The licking latency 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; °  $P < 0.05$ , °°  $P < 0.01$  in comparison with ryanodine (0.1 nmol per mouse i.c.v.), °°°  $P < 0.05$  in comparison with ryanodine (1 nmol per mouse i.c.v.); ^  $P < 0.05$  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-myoinositol, but not by L-myoinositol, used as negative control, confirming that the heparin effect observed

in the present study was due to the blockade of  $\text{InsP}_3$  receptors.

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

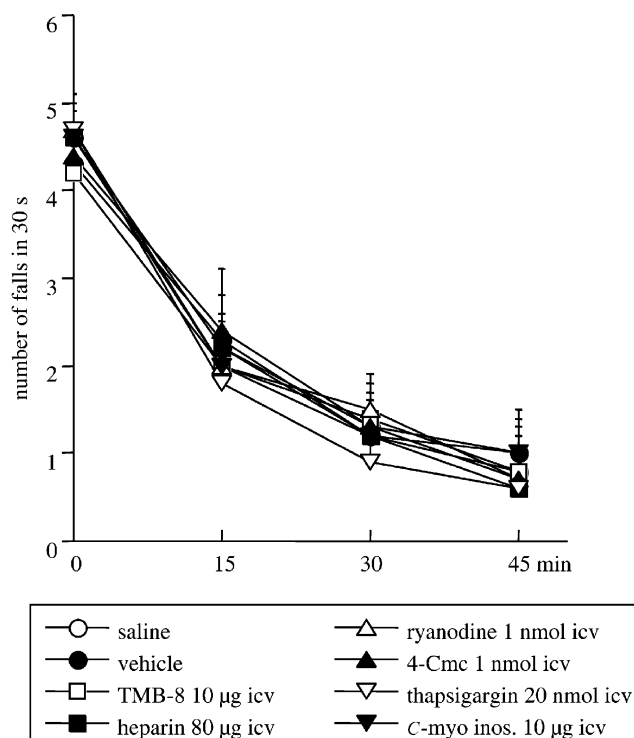


Fig. 7. Lack of effect by TMB-8 (10 µg per mouse i.c.v.), heparin (80 µg per mouse i.c.v.), ryanodine (1 nmol per mouse i.c.v.), 4-Cmc C (1 nmol per mouse i.c.v.), thapsigargin (20 nmol per mouse i.c.v.) and D-myoinositol (10 µg per mouse i.c.v.) on motor coordination in the mouse rota-rod test. Vertical lines represent S.E.M. 4-Cmc, thapsigargin and D-myoinositol 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.



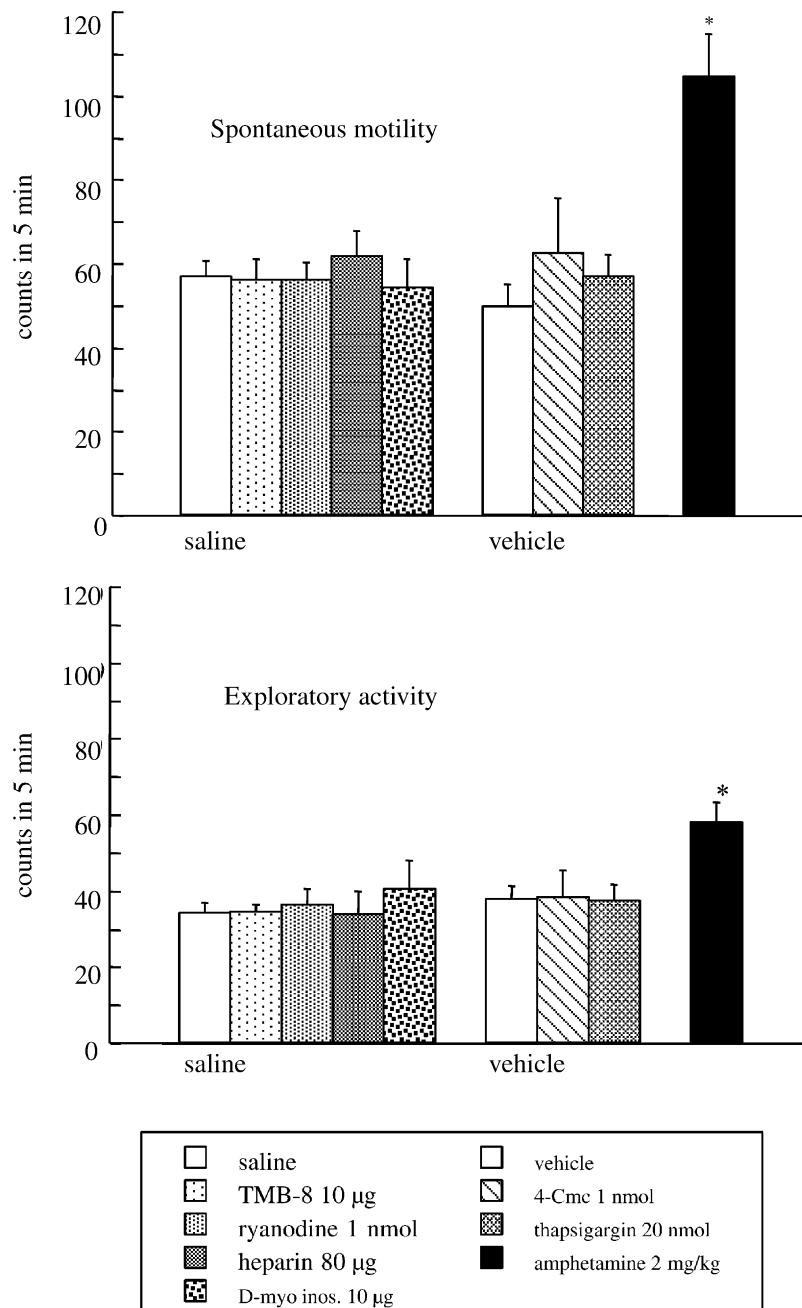


Fig. 8. Lack of effect by TMB-8 (10 µg per mouse i.c.v.), heparin (80 µg per mouse i.c.v.), ryanodine (1 nmol per mouse i.c.v.), 4-Cmc C (1 nmol per mouse i.c.v.), thapsigargin (20 nmol per mouse i.c.v.) and D-myo inositol (10 µg per mouse i.c.v.) on spontaneous motility and exploratory activity in the 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  $\text{Ca}^{2+}$  release from  $\text{Ca}^{2+}$ /caffeine-sensitive microsomal pools, which are involved in the phenomenon of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (McPherson et al., 1991). Ryanodine reduces the rate at which  $[\text{Ca}^{2+}]_i$  increase with  $\text{Ca}^{2+}$  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  $[\text{Ca}^{2+}]_i$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  regulates numerous neuronal functions (Berridge, 1998) and, therefore, a variation of intracellular  $\text{Ca}^{2+}$  contents can induce behavioral side effects. All the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  contents is involved in the modulation of acute thermal pain perception at the supraspinal level. In particular, an increase of intracellular  $\text{Ca}^{2+}$  enhances the pain threshold whereas a decrease of cytosolic  $\text{Ca}^{2+}$  renders animal hypersensitive to a thermal painful stimulus. Furthermore, both  $\text{InsP}_3\text{R}$  and RyR appear to participate to the modulation of acute thermal pain perception.

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