Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 567 (2007) 198–205
www.elsevier.com/locate/ejphar

Antinociceptive action of myricitrin: Involvement of the K^+ and Ca^{2+} channels

Flavia Carla Meotti [a,c], Roselei Fachinetto [a], Liana C. Maffi [c], Fabiana Cristina Missau [b], Moacir Geraldo Pizzolatti [b], João B.T. Rocha [a], Adair R.S. Santos [c,*]

[a] Departamento de Química, Universidade Federal de Santa Maria, 97110-000, Santa Maria, RS, Brazil
[b] Departamento de Química, Universidade Federal de Santa Catarina, Florianópolis 88040-900, SC, Brazil
[c] Departamento de Ciências Fisiológicas, Universidade Federal de Santa Catarina, Florianópolis 88040-900, SC, Brazil

Received 28 November 2006; received in revised form 15 March 2007; accepted 19 March 2007
Available online 30 March 2007

Abstract

The present study was designed to investigate the mechanisms involved in the antinociception afforded by myricitrin in chemical models of nociception in mice. Myricitrin given by intrathecal (i.t.) or intracerebroventricular (i.c.v.) route produced dose-related antinociception when evaluated against acetic acid-induced visceral pain in mice. In addition, the intraperitoneal administration of myricitrin caused significant inhibition of biting behaviour induced by i.t. injection of glutamate, substance P, capsaicin, interleukin 1 β (IL-1β) and tumor necrosis factor-α (TNF-α). The antinociception caused by myricitrin in the acetic acid test was fully prevented by i.t. pre-treatment with pertussis toxin, a Gi/o protein inactivator, and by i.c.v. injection of calcium chloride ($CaCl_2$). In addition, the i.t. pre-treatment of mice with apamin, a blocker of small (or low)-conductance calcium-gated K^+ channels and tetraethylammonium, a blocker of voltage-gated K^+ channels significantly reversed the antinociception induced by myricitrin. The charybdotoxin, a blocker of large (or fast)-conductance calcium-gated K^+ channels and glibenclamide, a blocker of the ATP-gated K^+ channels had no effect on myricitrin-induced antinociception. Calcium uptake analysis revealed that myricitrin inhibited $^{45}Ca^{2+}$ influx under a K^+-induced depolarization condition. However, calcium movement was modified in a non-depolarizing condition only when the highest concentration of myricitrin was used. In summary, our findings indicate that myricitrin produces consistent antinociception in chemical models of nociception in mice. These results clearly demonstrate an involvement of the Gi/o protein dependent mechanism on antinociception caused by myricitrin. The opening of voltage- and small-conductance calcium-gated K^+ channels and the reduction of calcium influx led to the antinociceptive of myricitrin.

Keywords: Myricitrin; Antinociception; Calcium movement; K^+ channel; Protein $G_{i/o}$

1. Introduction

Tissue damage results in release of mediators like excitatory amino acids, peptides, protons, lipids and cytokines, which bind to receptors (nociceptors) and activate signalling routes, including protein kinases A and C (PKA and PKC), calcium/calmodulin-dependent protein kinase, nitric oxide (NO) and mitogen-activated protein kinases (MAPKs) (Ji and Strichartz, 2004). As a result, the sensitization of primary afferent fibres transmits pain messages to the dorsal horn neurons and, subsequently, to the higher brain centre, resulting in the establishment of pain (Basbaum and Jessell, 2000; Julius and Basbaum, 2001). Taking this into account, substances able to counteract these signalling pathways at either a peripheral or central level, might be promising compounds to control pain.

We have previously demonstrated that myricitrin (Fig. 1), a naturally occurring flavonoid that is widespread in higher plants, comprises antinociceptive activity in chemical acute models of nociception (Meotti et al., 2006b). Myricitrin reduced paw edema and allodynia caused by intraplantar injection of complete Freund's adjuvant (CFA), as well the allodynia caused by sciatic partial constriction in mice (Meotti et al., 2006a). The mechanisms involved in the antinociceptive action of myricitrin include

* Corresponding author. Departamento de Ciências Fisiológicas, Universidade Federal de Santa Catarina, Campus Universitário — Trindade, 88040-900, Florianópolis, SC, Brazil. Tel.: +55 48 3721 9352/9444; fax: +55 48 3721 9672.
E-mail address: arssantos@ccb.ufsc.br (A.R.S. Santos).

0014-2999/$ - see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.ejphar.2007.03.039

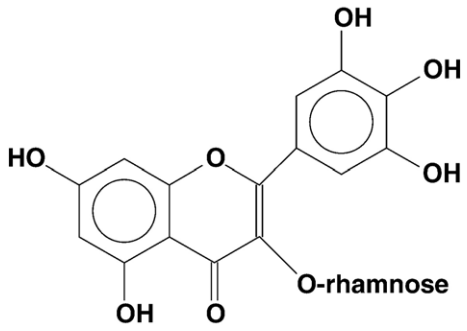

Fig. 1. Chemical structure of myricitrin.

inhibition of PKC, PI 3-kinase (PI 3K), nitric oxide (NO) production, nuclear factor-κB (NF-κB) activation, anti-inflammatory activity (Agullo et al., 1997; Gamet-Payrastre et al., 1999; Chen et al., 2000; Meotti et al., 2006b,a) and inhibition of p38 MAPK phosphorylation (unpublished data). The pathways of the antinociceptive effects of myricitrin are presented in the Fig. 2.

It is known that flavonoids interact with enzymes and proteins within cells and thus, regulate processes such as cell growth, apoptosis, immunological response and inflammation (Gamet-Payrastre et al., 1999; Middleton et al., 2000; Havsteen, 2002; Calixto et al., 2003; 2004; Ko et al., 2005). The present study was designed to investigate the effect of myricitrin on pathways of pain transmission, others than those previously described. The inhibitory signalling pathway, including protein $G_{i/o}$ activation and opening of potassium channels, as well excitatory signalling pathway, including calcium intracellular increase and those ruled by mediators as glutamate, substance P, capsaicin and pro-inflammatory cytokines (TNF-α and IL-1β) were evaluated. Finally, we investigated whether the antinociceptive effects of myricitrin could be extended to the central nervous system. In this experiment, we employed the acetic acid-induced visceral pain after intrathecal (i.t.) and intracerebroventricular (i.c.v.) injection of myricitrin.

2. Materials and methods

2.1. Animals

Experiments were conducted using adult Swiss mice (25–35 g) of both sexes and adult male Wistar rats (200–250 g), housed at 22±2 °C under a 12-h light/12-h dark cycle (lights on at 6:00) and with access to food and water *ad libitum*. Male and female mice were homogeneously distributed among groups, and all animals were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. The experiments were performed after approval of the protocol by the Institutional Ethics Committee and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals as specified by Zimmermann (1983).

2.2. Abdominal constriction induced by acetic acid

The abdominal constrictions were induced according to procedures described previously (Collier et al., 1968) and resulted in the contraction of the abdominal muscle together with a stretching of the hind limbs in response to an intraperitoneal (i.p.) injection of acetic acid (0.6%, 0.45 ml/mouse) at the time of the test. Mice were lightly anesthetized with ether and a volume of 5 μl of sterile phosphate buffer saline (PBS) or myricitrin (0.1–10 μg/site) was injected directly into the lateral ventricle (i.c.v.; coordinates from bregma: 1 mm lateral, 1 mm rostral, 3 mm vertical) or between the L5 and L6 vertebrae (intrathecal, i.t.) using a microsyringe connected to polyethylene tubing, as described previously (Laursen and Belknap, 1986; Hylden and Wilcox, 1980). The mice were treated with PBS or myricitrin 10 min before acetic acid injection. After the challenge, the mice were individually placed into glass cylinders of 20 cm diameter, and the abdominal constrictions were counted cumulatively over a period of 20 min.

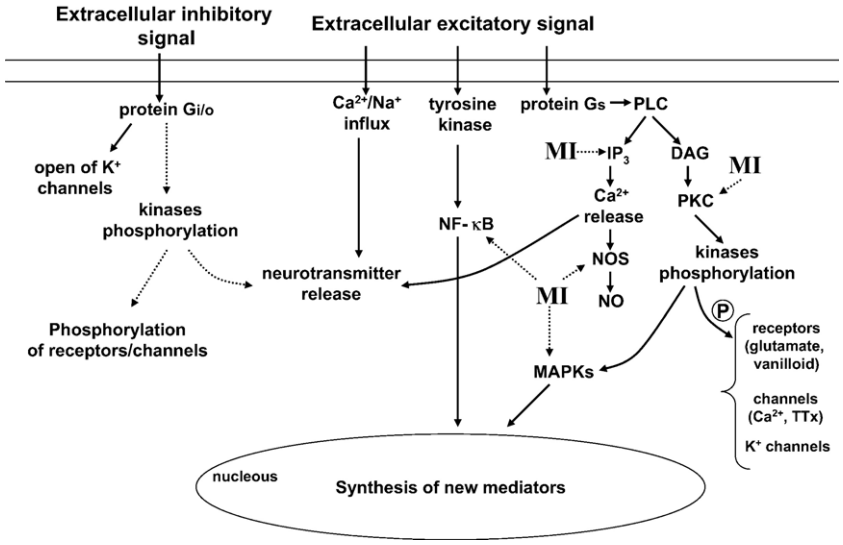

Fig. 2. Pain transmission pathways and myricitrin sites of action. The continuous arrow (→) shows the activation effect and the dot arrow (⋯▶) shows the inhibitory effect. MI (myricitrin); TTx (sodium channel tetrodotoxin resistant); K^+ (potassium ion); Na^+ (sodium ion); Ca^{2+} (calcium ion); PLC (phospholipase C); IP_3 (inositol triphosphate) PKC (protein kinase C); DAG (diacylglycerol); NOS (nitric oxide synthase); NO (nitric oxide); NF-κB (nuclear factor-κB); MAPKs (mitogen-activate protein kinases).

2.3. Algogen-induced overt nociception in mice

In another set of experiments we examined myricitrin effects on the nociception induced by glutamate, substance P, capsaicin and pro-inflammatory cytokines. Animals received an i.p. injection of myricitrin (30 mg/kg) 30 min before i.t. injection of 5 μl of drug. Injections were given to fully conscious mice awake using the method described by Hylden and Wilcox (1980). Briefly, the animals were restrained manually and a 30 gauge needle, attached to a 50 μl microsyringe, was inserted through the skin and between the vertebrae into the subdural space of the L5–L6 spinal segments. Injections were given over a period of 5 s. The nociceptive response was elicited by glutamate (30 μg/site) (Scheidt et al., 2002); substance P (135 ng/site) (Sakurada et al., 1990); capsaicin (30 ng/site) (Sakurada et al., 1996); IL-1β (1 pg/site) and TNF-α (0.1 pg/site) (Choi et al., 2003) with minor modifications. A group of mice received vehicle (PBS) by i.t. route. The amount of time the animal spent biting was evaluated following local post-injections of one of the following agonists: glutamate (3 min); substance P and capsaicin (6 min); IL-1β, TNF-α and PBS (15 min). A bite was defined as a single head movement directed at the flanks or hind limbs, resulting in contact of the animal's snout with the target organ.

2.4. Analysis of the mechanism of action of myricitrin

In this set of experiment myricitrin was administered intraperitoneally in mice. This provides a wide distribution of the compound that allows the evaluation of the effects of myricitrin at peripheral and central levels.

2.4.1. Participation of $G_{i/o}$ protein

To determine the involvement of $G_{i/o}$ protein in the antinociceptive action of myricitrin, mice were pre-treated with pertussis toxin (0.5 μg/site), an inactivator of $G_{i/o}$ protein. A control group was pre-treated with PBS (5 μl /site) by intrathecal route. The experiment was carried out as described by Sánchez-Blázquez and Garzón (1991). Seven days after the pre-treatment, mice received vehicle (10 ml/kg), myricitrin (1 mg/kg, i.p.) or morphine (2.5 mg/kg, s.c.) (Santos et al., 1999). After 30 min, the animals were injected with 0.6% acetic acid. The number of abdominal constrictions was recorded during the 20 min following acetic acid administration.

2.4.2. Involvement of K^+ channels in the antinociceptive action of myricitrin

We next investigated the involvement of K^+ channels on the antinociceptive action of myricitrin. Mice were pre-treated with K^+ channels blockers: apamin (50 ng/site, i.t.; a blocker of small (or low)-conductance calcium-gated K^+ channels); charybdotoxin (250 pg/site, i.t.; a blocker of large (or fast)-conductance calcium-gated K^+ channels); tetraethylammonium (1 μg/site, i.t.; a blocker of voltage-gated K^+ channels); or glibenclamide (80 μg/site, i.t.; a blocker of ATP-gated K^+ channels) and after 15 min they received myricitrin (1 mg/kg, i.p.), morphine (2.5 mg/kg, s.c.) or vehicle (10 ml/kg, i.p.) (Strong, 1990; Aronson, 1992; Welch and Dunlow, 1993; Santos et al., 1999). The nociceptive response was evaluated through the number of abdominal constrictions in 20 min, which was caused by an i.p. injection of acetic acid (0.6%, 0.45 ml) 30 min after myricitrin, morphine or vehicle administration.

2.4.3. Involvement of calcium channels in the antinociceptive action of myricitrin

Mice received 5 μl of $CaCl_2$ (200 nmol/site) or PBS (vehicle) by i.c.v. route as described by Liang et al. (2003). The i.c.v. injection was carried out as described above (Section 2.3). After 10 min animals were treated with myricitrin (1 mg/kg, i.p.), morphine (2.5 mg/kg, s.c.) or saline (10 ml/kg). Thirty min following drugs administration, the animals received 0.45 ml of acetic acid (0.6%) by i.p. route, and the number of abdominal constrictions was recorded over 20 min.

2.4.4. Modulation of $^{45}Ca^{2+}$ influx by myricitrin

To investigate myricitrin effects on calcium movement, the $^{45}Ca^{2+}$ influx into cortical slices of rats was assessed. $^{45}Ca^{2+}$ uptake was carried out essentially as described by Eason and Aronstam (1984), with some modifications. Two salt solutions were used in these studies: (1) Krebs buffer containing 127 mM NaCl, 1.2 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.95 mM $MgCl_2$, 0.70 mM $CaCl_2$, 10 mM glucose, and 0.50 mM Hepes, pH 7.4 with KCl 5.36 mM for a baseline analysis or 80 mM for the K^+-stimulated assay; (2) lanthanium solution containing 127 mM NaCl, 0.95 mM $MgCl_2$, 10 mM $LaNO_3$, 10 mM glucose, and 0.60 mM Hepes, pH 7.4 with KCl 5.0 mM for a baseline analysis or 80 mM for the K^+-stimulated assay. To measure $^{45}Ca^{2+}$ uptake, rats were killed by decapitation, the cerebral cortex was dissected, isolated and the parietal cortex was cut into 400 μm slices, which were washed with Krebs buffer (solution 1). The slices (0.8–1.3 mg protein) were pre-incubated in 96-well polycarbonate plates for 22 min at 32 °C in the absence (control group) or presence of myricitrin (100 and 200 μM). The slices were then transferred to medium containing solution 1 plus 21 pmol of $^{45}Ca^{2+}$. The $^{45}Ca^{2}$ uptake was monitored for 15 s at 32 °C. The reaction was stopped by five times of 2 min washes with ice-cold lanthanium solution (solution 2). Immediately after washing, aliquots were lysed with 0.25 ml of a solution containing 0.5 M NaOH plus 0.2% SDS and maintained at 60 °C for 5 min. An aliquot was taken for determination of the intracellular calcium content by liquid scintillation counting. Nonspecific calcium uptake (20–30% of the total uptake) was determined by carrying out the same experiment using solution 2, which contained the nonspecific voltage-dependent calcium channel blocker, lanthanium. Specific uptake was considered as the difference between total uptake and nonspecific uptake.

2.5. Protein measurement

The protein content of the synaptic membrane preparations and of cortical slices was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Table 1
Effect of supraspinal, spinal or systemic administration of myricitrin on acetic acid-induced visceral pain

	ID_{50}	MI (%)
Myricitrin i.c.v. (μg/site)	5.79 (2.04–16.41)	57±5
Myricitrin i.t. (μg/site)	3.32 (1.53–7.2)	82±9
Myricitrin i.p. (mg kg^{-1})[a]	0.33 (0.2–0.54)	84±5

The mice received 5 μl of myricitrin into intracerebroventricular (i.c.v.), intratecal (i.t.) (0.1 – 10 μg/site) or intraperitoneal (i.p.) (0.01–10 mg/kg). Ten minutes after i.c.v. or i.t. and 30 min after i.p. administration the mice received 0.45 ml of i.p. acetic acid (0.6%). Immediately after acetic acid administration the amount of abdominal constriction was summed in 20 min. The ID_{50} values (*i.e.*, dose of myricitrin that inhibits 50% of acetic acid response) are reported as geometric means accompanied by their respective 95% confidence limits, using three dosages of myricitrin (n=6 per group). The percentage of maximal inhibition (MI %) were calculated at 10 μg/site, by i.c.v. and i.t. routes or 10 mg/kg by i.p. route.
[a] Data from Meotti et al., 2006b.

2.6. Materials

The following substances were used: morphine hydrochloride and acetic acid (Merck, Darmstadt, Germany); capsaicin, glutamic acid, cytokines (tumor necrosis factor-α and interleukin-1β), calcium chloride, charybdotoxin, tetraethylammonium, apamin and phosphate-buffered saline (Sigma, St. Louis, USA); glibenclamide, and substance P (Tocris Cookson Inc., Ellisville, USA); $^{45}Ca^{2+}$ was purchased from Amersham International, UK. All other chemicals were of analytical grade and obtained from standard commercial suppliers. Morphine was dissolved in 0.9% NaCl solution and the other drugs were dissolved in phosphate-buffered saline pH 7.0 (PBS), with the exception of myricitrin and glibenclamide, which were dissolved in Tween 80 plus saline, and capsaicin, which was dissolved in alcohol plus saline. The final concentration of Tween and alcohol did not exceed 5% and did not cause any effect *per se*. The myricitrin was isolated from genus Eugenia at the Department of Chemistry, Universidade Federal de Santa Catarina, Brazil. It was identified by spectral analyses (RMN-1H) and (RMN-13C) and by comparison with the spectrum literature data (Agrawal, 1989) and showed a degree of purity greater than 98%.

2.7. Statistical analysis

The results are presented as mean (S.E.M.), except for the ID50 values (*i.e.*, the dose of myricitrin reducing the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The ID50 value was determined using three doses of myricitrin by linear regression from individual experiments using linear regression software (GraphPad software, San Diego, CA). Percentage of maximal inhibition was calculated at the most effective dose used. Data were analyzed by one-way ANOVA followed by Newman–Keuls test, with the exception of the in vitro test of calcium uptake, which was analyzed by two-way ANOVA followed by Newman–Keuls test (potassium pre-treatment and myricitrin treatment). The statistical differences were considered significant when $P<0.05$.

3. Results

3.1. Spinal and supraspinal antinociception of myricitrin

The results given in Table 1 show that myricitrin, administered by i.c.v. or i.t. route 10 min prior to testing, produced dose-related inhibition of the acetic acid-induced abdominal constrictions in mice. The ID_{50} data (and their respective 95% confidence limits) and inhibition percentage, at a dose of 10 μg/site, are shown in Table1. These results together with previous results (Meotti et al., 2006b) have demonstrated that myricitrin is very effective in decreasing nociception induced by acetic acid when given by supraspinal (i.c.v.), spinal (i.t.) or systemic (i.p.) routes.

3.2. Algogen-induced overt nociception in mice

Intrathecal administration of glutamate, capsaicin, substance P, IL-1β and TNF-α caused significant biting behaviour in mice when compared to animals injected intrathecally with PBS ($P<0.001$) (Fig. 3). The results (seconds) for the biting behaviour in mice pre-treated with saline i.p. were: 7.7±3.6 for PBS; 183.1±21.3 for glutamate; 106.2±5.1 for capsaicin; 89.2±6.6 for substance P; 116.1±20.8 for IL-1β and 124.1±17.0 for TNF-α. In all groups, systemic pre-treatment with myricitrin (30 mg/kg, i.p.) significantly reduced the biting behaviour when compared with mice treated with saline i.p. The greatest effect of myricitrin appeared against cytokines where the inhibition percentages were: TNF-α (100%); IL-1β (96±2%); substance P (84±10%); capsaicin (61±9%) and glutamate (41±13%) (Fig. 3).

3.3. $G_{i/o}$ protein participation

The results illustrated in Fig. 4 show that inactivation of the $G_{i/o}$ protein, which was caused by an i.t. injection of pertussis toxin 7 days before testing (Sánchez-Blázquez and Garzón, 1991), completely abolished the antinociceptive effect of myricitrin

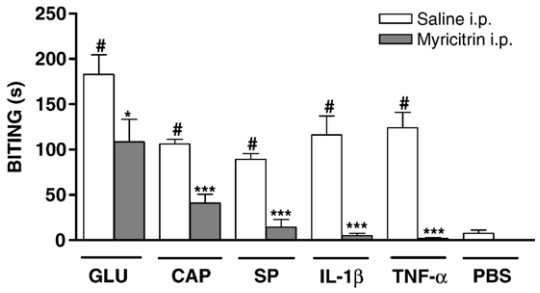

Fig. 3. Effects of myricitrin (30 mg/kg, i.p.) on the nociceptive behaviour induced by glutamate (GLU 35 μg/site); capsaicin (CAP 30 ng/site); substance P (SP 135 ng/site); interleukin-1β (IL-1β 1 pg/site); tumor necrosis factor-α (TNF-α 0.1 pg/site) or phosphate buffer saline (PBS 5 μl/site). Each column represents the mean of six mice and the error bars indicate the S.E.M. in the absence (white column, control groups) or in the presence (gray column) of myricitrin. The symbols denote significance levels: *$P<0.05$ and ***$P<0.001$ compared to their respective saline treated group, $^{\#}P<0.001$ from PBS group, one-way ANOVA followed by Newman–Keuls test.

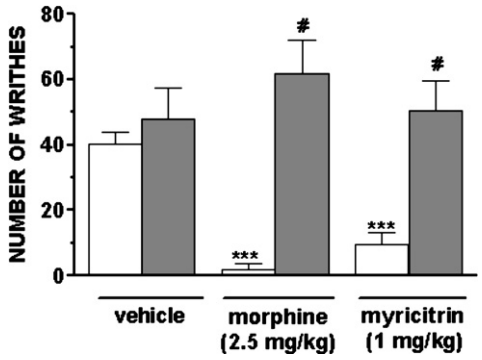

Fig. 4. Effect of pre-treatment (seven days) with pertussis toxin (0.5 μg/site, i.t.) on the antinociceptive profiles of myricitrin (1 mg/kg, i.p.) and morphine (2.5 mg/kg, s.c.) against the acetic acid-induced writhing in mice. Each column represents the mean of six to eight mice and the error bars indicate the S.E.M. in the absence (white column) or presence (gray column) of pertussis toxin. The symbols denote significance levels: ***$P<0.001$ compared to control group; $^{\#}P<0.001$ compared to group treated with morphine or myricitrin, one-way ANOVA followed by Newman–Keuls test.

(1 mg/kg). In addition, we have found that antinociception caused by morphine (2.5 mg/kg, s.c.) was completely abrogated by i.t. injection of pertussis toxin. Additionally, the pre-treatment with pertussis toxin did not cause any effect *per se* on acetic acid-induced abdominal constriction. In this situation, we can identify an important role of the $G_{i/o}$ protein in the antinociceptive effect of myricitrin.

3.4. Involvement of voltage- and small-conductance Ca^{2+}-gated K^+ channels in the antinociceptive action of myricitrin

The results in Fig. 5 show that intrathecal pre-treatment with tetraethylammonium and apamin significantly reversed the antinociceptive effect of myricitrin. On the other hand, glibenclamide and charybdotoxin did not reverse myricitrin effect (Fig. 5). However, unexpectedly the treatment of animals with charybdotoxin, at the same dose, produced a synergic effect with myricitrin. In addition, all blockers prevented the morphine antinociception (positive control). Given alone, K^+ channel blockers did not produce any effect on the acetic acid-induced visceral pain (Fig. 5).

3.5. Involvement of Ca^{2+} channels in the antinociceptive action of myricitrin

The results in Fig. 6 show that pre-treatment with $CaCl_2$, by i.c.v. route, fully prevented the antinociceptive effect of myricitrin on acetic acid-induced visceral pain. Furthermore, the same pre-treatment completely prevented morphine-induced antinociception. However, $CaCl_2$ alone did not have any effect on acetic acid-induced nociception.

3.6. $^{45}Ca^{2+}$ influx

The results depicted in Fig. 7 show myricitrin effects upon $^{45}Ca^{2+}$ uptake into cortical slices from rats. In this assay, the

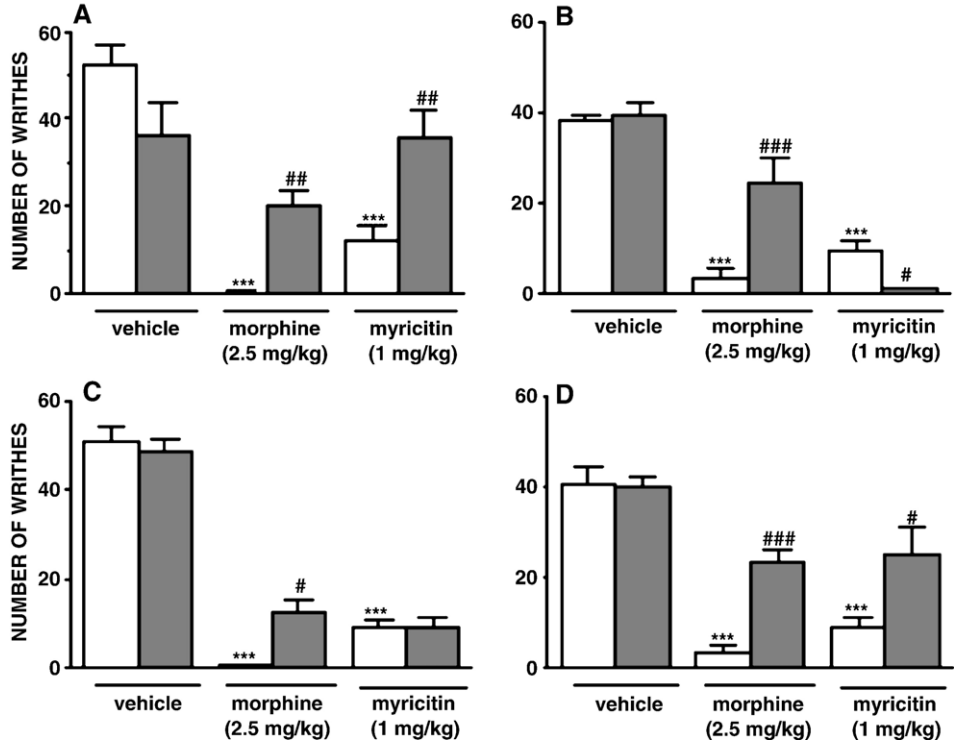

Fig. 5. Effect of i.t. pre-treatment (seven days) with (A) tetraethylammonium (1 μg/site); (B) charybdotoxin (250 pg/site); (C) glibenclamide (80 μg/site) and (D) apamin (50 ng/site) on the antinociceptive profile of myricitrin (1 mg/kg, i.p.) against the acetic acid-induced writhing in mice. Each column represents the mean of six mice and the error bars indicate the S.E.M. in the absence (white column) or presence (gray column) of K^+ channel blockers. The symbols denote significance levels: ***$P<0.001$ compared to control group; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared to myricitrin or morphine group, $^{\#\#\#}P<0.001$ compared to morphine group, one-way ANOVA followed by Newman–Keuls test.

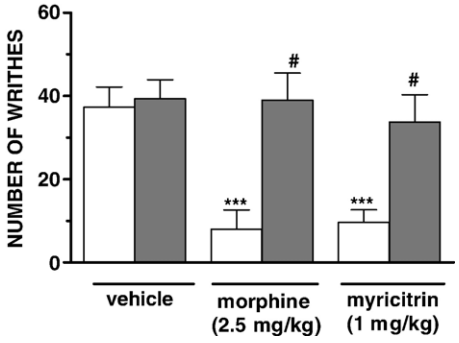

Fig. 6. Effect of pre-treatment with $CaCl_2$ (200 nmol/site, i.c.v.) on the antinociceptive profiles of myricitrin (1 mg/kg, i.p.) and morphine (2.5 mg/kg, s.c.) against the acetic acid-induced writhing in mice. Each column represents the mean of six mice and the error bars indicate the S.E.M. in the absence (white column) or presence (gray column) of $CaCl_2$. The symbols denote significance levels: ***$P<0.001$ compared to control group; $^{\#}P<0.001$ compared to group treated with morphine or myricitrin, one-way ANOVA followed by Newman–Keuls test.

medium containing the higher K^+ concentration (a depolarizing condition) led to an increase of 30.0±4.3% ($P<0.05$) in $^{45}Ca^{2+}$ uptake when compared to medium with lower K^+ concentration (non-depolarizing condition). Myricitrin at 100 and 200 μM inhibited $^{45}Ca^{2+}$ uptake in a K^+-induced depolarizing condition ($P<0.001$). The percentages of inhibition were 46±13% and 48±11% for 100 and 200 μM, respectively. Alternatively, in the absence of a depolarizing condition, only the highest concentration of myricitrin was able to reduce $^{45}Ca^{2+}$ influx (P<0.01), where the percentage of inhibition was 42±2%. Through two-way analyses we detected a significant interaction between the presence of myricitrin and a K^+-induced depolarizing condition [$F(2,12)=4.54$ ($P<0.05$)], suggesting that inhibitory effect of myricitrin on calcium movement is more pronounced when the cell is stimulated.

4. Discussion

A considerable number of studies have reported flavonoids exert antioxidant, anti-inflammatory, immunomodulatory, anti-allergic, neuroprotective, anti-mutagenic, anti-rheumatic and antinociceptive effects (Havsteen., 2002; Calixto et al., 2003, 2004; Meotti et al., 2006b). In previous studies, we have demonstrated that the systemic administration of myricitrin reduced both acute and chronic pain in mice. The antinociceptive action of this flavonoid has been attributed to inhibition of protein kinase activity, NO production and anti-inflammatory activity (Agullo et al., 1997; Gamet-Payrastre et al., 1999; Chen et al., 2000; Meotti et al., 2006b,a).

Since myricitrin has been administered by systemic (i.p.) route, its antinociceptive effects could occur by peripheral or central sites of action. In addition, the acetic acid-induced abdominal constriction, which is the model of pain employed in this and previous studies with myricitrin, activates both central and peripheral sites of pain (Collier et al., 1968; Vineger et al., 1979; Ribeiro et al., 2000; Ikeda et al., 2001; Julius and Basbaum, 2001; Feng et al., 2003). To distinguish the site of action of myricitrin, we administered it directly into spinal and supraspinal locations. Thus, the rise of the nociceptive threshold caused by spinal and supraspinal administration of myricitrin demonstrated that antinociceptive effect of this flavonoid is extended to central next to peripheral level.

Glutamate and the neuropeptide substance P are the main mediators responsible for pain transmission in the dorsal horn (Sakurada et al., 1990; Liu et al., 1997; Ji and Strichartz, 2004; Caruso et al., 2005). It is well established that these substances, together with the vanilloid receptor agonist capsacin, promote neuronal excitability in dorsal horn through mechanisms that involve NO production and PKC activation (Sakurada et al., 1996; Liu et al., 1997; Ji and Strichartz, 2004; Caruso et al., 2005). Regarding inhibitory effects of myricitrin against NO production and PKC activation, we tested myricitrin effects against biting behaviour induced by glutamate; substance P and capsaicin. Our results demonstrated that myricitrin systemic administration significantly reduced the biting induced by spinal administration of these different mediators. In line with this, it is likely that the antinociceptive action of myricitrin on the glutamate, substance P and capsaicin-induced nociception is associated with its ability to reduce NO level and PKC activity (Gamet-Payrastre et al., 1999; Chen et al., 2000; Meotti et al., 2006b).

The present study demonstrated that myricitrin, given intraperitoneally, produces a strong inhibition of the biting response caused by i.t. injection of cytokines, IL-1β and TNF-α. Cytokines were reported to induce biting behaviour when administered intrathecally in mice (Choi et al., 2003). The binding of IL-1β to its receptor IL-1RI actives tyrosine kinases and calcium-independent PKC (Obreja et al., 2002; Sommer and Kress, 2004), which could support nociception caused by this peptide. In agreement, we have reported previously, through *in vivo* and *in vitro* studies, that myricitrin inhibits nociception mainly by its property of PKC inhibition (Meotti et al., 2006b). These results together, strongly suggest that myricitrin effects on IL-1β-induced nociception are due to its ability to inhibit PKC.

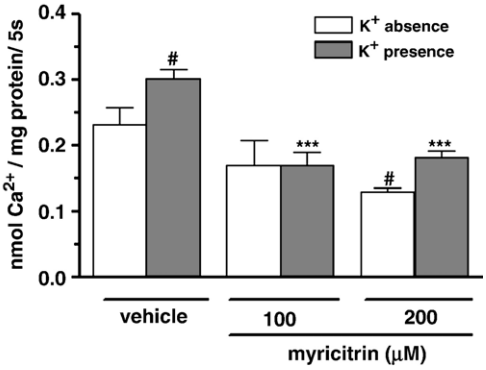

Fig. 7. Effect of myricitrin on the calcium influx into rat cerebral cortical slices. Rat cerebral cortical slices were incubated with 21 pmol of $^{45}Ca^{2+}$ in the presence or absence (control groups) of myricitrin (100 and 200 μM). The assays were carried out under non-depolarizing conditions (white column) and under depolarizing conditions (gray column). Values are the means of three experiments with each experimental value being the average of three replicates. Data are expressed as mean±S.E.M. and the symbols denote significance levels: $^{\#}P<0.01$ compared to control group (non-depolarizing conditions); ***$P<0.001$ compared to control group (depolarizing conditions), two-way ANOVA followed by Newman–Keuls test.

Our results demonstrate that myricitrin completely inhibited TNF-α-induced nociception. Previous reports show that TNF-α-induced nociception involves phosphorylation of p38 MAPK (Schäfers et al., 2003). In addition, myricitrin potently inhibited p38 MAPK phosphorylation on the spinal medulla (unpublished data). In line with this, myricitrin may inhibit TNF-α-induced nociception by decreasing phosphorylation of intracellular signalling kinases. Alternatively, the antinociceptive action of myricitrin on TNF-α-induced biting behaviour may be due to myricitrin effects upon calcium channels, since recent reports showed that TNF-α activates Ca^{2+} mobilization in cultured sensory neurons (Pollock et al., 2002).

The present study also demonstrated that the inactivation of the $G_{i/o}$ protein, through i.t. pertussis toxin treatment, completely prevented the antinociception induced by systemic administration of myricitrin. Activation of $G_{i/o}$ protein is considered an inhibitory signalling event, since it inhibits adenylyl cyclase and opens two different types of K^+ inward rectifier (K_{ir}) channels: K^+-ATP dependent and G-protein-regulated inwardly rectifying K^+ (GIRK) channels (Childers and Deadwyler, 1996; Ocaña et al., 2004). Considering glibenclamide did not avert antinociception of myricitrin, it seems unlikely that myricitrin effects are due to the opening of the K^+-ATP dependent channel. On the other hand, we can not exclude an effect of the myricitrin upon GIRK channels.

The pre-treatment with tetraethylammonium, a blocker of voltage-gated K^+ channels and apamin, a blocker of small (or low)-conductance calcium-gated K^+ channels reversed myricitrin antinociceptive effect. However, the pre-treatment with charybdotoxin, a blocker of large (or fast)-conductance calcium-gated K^+ channels did not prevent the myricitrin action. Therefore, myricitrin seems to exert specific action upon K^+ channels responsive to voltage- and K^+ channels of small-conductance calcium-gated. The difference between small and large-conductance calcium-gated K^+ channels resides in the intracellular C-terminal domain. The small-conductance calcium-gated K^+ channels have a calmodulin-binding domain that allows the channel to interact with calmodulin and to be regulated by Ca^{2+}, while the large-conductance calcium-gated K^+ channels have a region termed the "calcium bowl", that binds directly to the Ca^{2+} (Ocaña et al., 2004). This structural difference could explain the different responsiveness of these channels to myricitrin. However, molecular studies are necessary to address this hypothesis.

Remarkably, calcium chloride pre-treatment fully prevented the antinociception induced by myricitrin and morphine. It is well established that an increase in intracellular Ca^{2+} represents a key step for neurotransmitter release, cell membrane excitability, activation of intracellular proteins and reduces the pain threshold (Prado, 2001; Cervero and Laird, 2003; Ward, 2004). Our *in vitro* assays, using rat cortical slices, demonstrated that myricitrin inhibited $^{45}Ca^{2+}$ influx under a K^+-induced depolarizing condition. However, myricitrin modified calcium movement in a non-depolarizing condition only at the highest concentration used. These results may indicate that myricitrin interacts in a distinct way with Ca^{2+} channels, depending on the state of cell depolarization. Considering that myricitrin-induced antinociception was prevented by calcium pre-treatment (*in vivo*) and that myricitrin altered calcium movement (*in vitro*), we suggest that the blocking of calcium channels contributes to the antinociceptive action of myricitrin. At this time, the putative mechanisms that explain the antinociceptive activity of the myricitrin are presented in the Fig. 8.

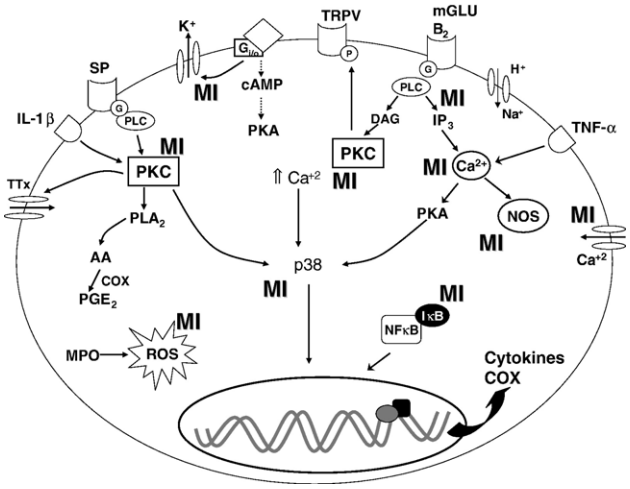

Fig. 8. Previous and news sites of action of myricitrin on the reduction of nociception. The continuous arrow (→) shows the activation effect and the dot arrow (⋯▶) shows the inhibitory effect. MI (myricitrin); TTx (sodium channel tetrodotoxin resistant); K^+ (potassium ion); Na^+ (sodium ion); Ca^{2+} (calcium ion); PLC (phospholipase C); IP_3 (inositol triphosphate); DAG (diacylglycerol); PKC (protein kinase C); PKA (protein kinase A); cAMP (cyclic adenosine monophosphate); NOS (nitric oxide synthase); NO (nitric oxide); NF-κB (nuclear factor-κB); p38 (mitogen-activate protein kinase);IL-1β (interleukin 1β); TNF-α (tumor necrosis factor α); SP (substance P); $G_{i/o}$ ($G_{i/o}$ protein); TRPV (transient receptor vanilloid); B_2 (metabotropic receptor for bradikinin); mGLU (metabotropic receptor for glutamatergic agonists); H^+ (proton); PLA_2 (phospholipase A_2); AA (araquidonic acid); COX (cyclooxygenase); MPO (myeloperoxidase); ROS (reactive oxygen species).

In conclusion, the present results are in agreement with previous data and demonstrate that myricitrin produces antinociception when administered at peripheral or central levels. Furthermore, these results showed that myricitrin antinociception is closely related to pathways activated by glutamate, substance P, capsaicin and pro-inflammatory cytokines. The mechanisms of antinociception are dependent of the $G_{i/o}$ protein activation; opening of specific K^+ channels (voltage- and small-conductance Ca^{2+}-gated) and inhibition of calcium influx.

Acknowledgements

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Programa de Apoio aos Núcleos de Excelência (PRONEX) and Fundação de Apoio a Pesquisa Científica e Tecnológica do Estado de Santa Catarina (FAPESC), Brazil. F.C. Meotti is a PhD student in Biochemical Toxicology; she thanks CAPES for the fellowship support.

The authors thank Dr. Anna Champman from Free Radicals Research Group, New Zealand, for the critical review of the manuscript.

References

Agrawal, P.K., 1989. Flavonoid glycosides. Studies in Organic Chemistry 39: Carbon-13 NMR of Flavonoids. Elsevier Science Publishers, New York, pp. 338–339.

Agullo, G., Gamet-Payrastre, L., Manenti, S., Viala, C., Rémésy, C., Chap, H., Payrastre, B., 1997. Relationship between flavonoid structure and inhibition of phosphatidylinositol-3 kinase: a comparison with tyrosine kinase and protein kinase C inhibition. Biochem. Pharmacol. 53, 1649–1657.

Aronson, J.K., 1992. Potassium channels in nervous tissue. Biochem. Pharmacol. 43, 11–14.

Basbaum, A.I., Jessell, T.M., 2000. In: Kandel, E.R., Schwartz, J.H., Jessell, T.M. (Eds.), Principles of Neuroscience. McGraw-Hill, New York, pp. 472–491.

Calixto, J.B., Otuki, M.F., Santos, A.R.S., 2003. Anti-inflammatory compounds of plant origin. Part I. Action on arachidonic acid pathway, nitric oxide and nuclear factor κB (NFκB). Planta Med. 69, 973–983.

Calixto, J.B., Campos, M.M., Otuki, M.F., Santos, A.R.S., 2004. Anti-inflammatory compounds of plant origin. Part II. Modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. Planta Med. 70, 93–103.

Caruso, C., Durand, D., Watanobe, H., Lasaga, M., 2005. NMDA and group I metabotropic glutamate receptors activation modulates substance P release from the arcuate nucleus and median eminence. Neurosci. Lett. 393, 60–64.

Cervero, F., Laird, J.M.A., 2003. Role of ions channels in mechanisms controlling gastrointestinal pain pathways. Curr. Opin. Pharmacol. 3, 608–612.

Chen, Y.C., Yang, L.L., Lee, T.J.F., 2000. Oroxylin A inhibition of lipopolysaccharide-induced iNOS and COX-2 gene expression via suppression of nuclear factor-κ B activation. Biochem. Pharmacol. 59, 1445–1457.

Childers, S.R., Deadwyler, S.A., 1996. Role of cyclic AMP in the actions of cannabinoid receptors. Biochem. Pharmacol. 52, 819–827.

Choi, S.S., Han, K.J., Lee, J.K., Lee, H.K., Han, E.J., Kim, D.H., Suh, H.W., 2003. Antinociceptive mechanisms of orally administered decursinol in the mouse. Life Sci. 73, 471–485.

Collier, H.O.J., Dinneen, J.C., Johnson, C.A., Schneider, C., 1968. The abdominal constriction response and its suppression by analgesic drugs in the mouse. Br. J. Pharmacol. Chemother. 32, 295–310.

Eason, K.E., Aronstam, R.S., 1984. Influence of sulfhydryl reagents on potassium-stimulated calcium uptake by rat brain synaptosomes. Res. Commun. Chem. Pathol. Pharmacol. 44, 503–506.

Feng, Y., Cui, M., Willis, W., 2003. Gabapentin markedly reduces acetic acid-induced visceral nociception. Anesthesiology 98, 729–733.

Gamet-Payrastre, L., Manenti, S., Gratacap, M.P., Tulliez, J., Chap, H., Payrastre, B., 1999. Flavonoids and the inhibition of PKC and PI 3-kinase. Gen. Pharmacol. 32, 279–286.

Havsteen, B.H., 2002. The biochemistry and medical significance of the flavonoids. Pharmacol. Ther. 96, 67–202.

Hylden, J.L., Wilcox, G.L., 1980. Intrathecal morphine in mice: a new technique. Eur. J. Pharmacol. 67, 313–316.

Ikeda, Y., Ueno, A., Naraba, H., Ohishi, S., 2001. Involvement of vanilloid receptor VR1 and prostanoids in the acid-induced writhing responses of mice. Life Sci. 69, 2911–2919.

Ji, R.R., Strichartz, G., 2004. Cell signalling and the genesis of neuropathic pain. Science 252, 1–19.

Julius, D., Basbaum, A.I., 2001. Molecular mechanisms of nociception. Nature 413, 203–210.

Ko, C.H., Shen, S.C., Hsu, C.S., Chen, Y.C., 2005. Mitochondrial-dependent, reactive oxygen species-independent apoptosis by myricetin: roles of protein kinase C, cytochrome *c*, and caspase cascade. Biochem. Pharmacol. 69, 913–927.

Laursen, S.E., Belknap, J.K., 1986. Intracerebroventricular injections in mice. Some methodological refinements. J. Pharmacol. Methods 16, 355–357.

Liang, J.H., Wang, X.H., Liu, R.K., Sun, H.L., Ye, X.F., Zheng, J.W., 2003. Buspirone-induced antinociception is mediated by L-type calcium channels and calcium/caffeine-sensitive pool in mice. Psychopharmacology 166, 276–283.

Liu, H., Mantayh, P.W., Basbaum, A.I., 1997. NMDA-receptor regulation of substance P release from primary afferent nociceptors. Nature 386, 721–724.

Lowry, O.H., Roseborough, N.R., Farr, A.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Meotti, F.C., Missau, F.C., Ferreira, J., Pizzolatti, M.G., Mizuzaky, C., Nogueira, C.W., Santos, A.R.S., 2006a. Anti-allodynic property of flavonoid myricitrin in models of persistent inflammatory and neuropathic pain in mice. Biochem. Pharmacol. 72, 1707–1713.

Meotti, F.C., Luiz, A.P., Pizzolatti, M.G., Kassuya, C.A.L., Calixto, J.B., Santos, A.R.S., 2006b. Analysis of the antinociceptive effect of the flavonoid myricitrin. Evidence for a role of the L-arginine–nitric oxide and protein kinase C pathways. J. Pharmacol. Exp. Ther. 316, 789–796.

Middleton, E., Kandaswami, C., Theoharides, T.C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol. Rev. 52, 673–751.

Obreja, O., Rathee, P.K., Lips, K.S., Distler, C., Kress, M., 2002. 1IL-1β potentiates heat-activated currents in rat sensory neurons: involvement of IL-1RI, tyrosine kinase and protein kinase C. FASEB J. 16, 1497–1503.

Ocaña, M., Cendán, C.M., Cobos, E.J., Entrena, J.M., Baeyens, J.M., 2004. Potassium channels and pain: present realities and future opportunities. Eur. J. Pharmacol. 500, 203–219.

Pollock, J., McFarlane, S.M., Connell, M.C., Zehavi, U., Vandenabeele, P., MacEwan, D.J., Scott, R.H., 2002. TNF-α receptors simultaneously activate Ca^{2+} mobilisation and stress kinases in cultured sensory neurons. Neuropharmacology 42, 93–106.

Prado, W.A., 2001. Involvement of calcium in pain and antinociception. Braz. J. Med. Biol. Res. 34, 449–461.

Ribeiro, R.A., Vale, M.L., Thomazzi, S.M., Paschoalato, A.B.P., Poole, S., Ferreira, S.H., Cunha, F.Q., 2000. Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. Eur. J. Pharmacol. 387, 111–118.

Sakurada, T., Manome, Y., Tan-No, K., Sakurada, S., Kisara, K., 1990. The effects of substance P analogues on the scratching, biting and licking response induced by intrathecal injection of *N*-methyl-D-aspartate in mice. Br. J. Pharmacol. 101, 307–310.

Sakurada, T., Sugiyama, A., Sakurada, C., Tanno, K., Sakurada, S., Kisara, K., Hara, A., Abiko, Y., 1996. Involvement of nitric oxide in spinally mediated capsaicin- and glutamate-induced behavioural response in the mouse. Neurochem. Int. 29, 271–278.

Sánchez-Blázquez, P., Garzón, J., 1991. Cholera toxin and pertussis toxin an opioid- and -α 2-mediated supraspinal analgesia in mice. Life Sci. 48, 1721–1727.

Santos, A.R.S., De Campos, R.O.P., Miguel, O.G., Cechinel-Filho, V., Yunes, R.A., Calixto, J.B., 1999. The involvement of K^+ channels and $G_{i/o}$ protein in the antinociceptive action of the gallic acid ethyl ester. Eur. J. Pharmacol. 379, 7–17.

Schäfers, M., Svensson, C.I., Sommer, C., Sorkin, L.S., 2003. Tumor necrosis factor-α induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. J. Neurosci. 23, 2517–2521.

Scheidt, C., Santos, A.R.S., Ferreira, J., Malheiros, A., Cechinel-Filho, V., Yunes, R.A., Calixto, J.B., 2002. Evidence for the involvement of glutamatergic receptors in the antinociception caused in mice by the sesquiterpene drimanial. Neuropharmacology 43, 340–347.

Sommer, C., Kress, M., 2004. Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. Neurosci. Lett. 361, 184–187.

Strong, P.N., 1990. Potassium channel toxins. Pharmacol. Ther. 46, 137–162.

Vineger, R., Truax, J.F., Selph, J.L., Johnston, P.R., 1979. Antagonism of pain and hyperalgesia. Anti-inflammatory drugs. In: Vane, J.R., Ferreira, S.H. (Eds.), Handbook of Experimental Pharmacology, vol. 50/II. Springer, Verlag, Berlin, pp. 208–222.

Ward, D.T., 2004. Calcium receptor-mediated intracellular signalling. Cell Calcium 35, 217–228.

Welch, S.P., Dunlow, L.D., 1993. Antinociceptive activity of intrathecally administered potassium channel openers and opioid agonists: a common mechanism of action? J. Pharmacol. Exp. Ther. 267, 390–399.

Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.