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Contribution of peripheral vanilloid receptor to the nociception induced by injection of spermine in mice

Camila Gewehr [a], Mariane Arnoldi da Silva [b], Gabriela Trevisan dos Santos [b], Mateus Fortes Rossato [b], Sara Marchesan de Oliveira [b], Carine Cristiane Drewes [d], Andréia Martini Pazini [b], Gustavo Petri Guerra [b], Maribel A. Rubin [a,b,c], Juliano Ferreira [a,b,c,*]

[a] Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[b] Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[c] Department of Chemistry, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[d] Programa de Pós-Graduação em Toxicologia, Universidade de São Paulo, São Paulo, SP, Brazil

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ABSTRACT

Polyamines (putrescine, spermidine and spermine) are important endogenous regulators of ion channels, such as vanilloid (TRPV1), glutamatergic (NMDA or AMPA/kainate) and acid-sensitive (ASIC) receptors. In the present study, we have investigated the possible nociceptive effect induced by polyamines and the mechanisms involved in this nociception *in vivo*. The subcutaneous (s.c.) injection of capsaicin (as positive control), spermine, spermidine or putrescine produced nociception with ED_{50} of 0.16 (0.07–0.39) nmol/paw, 0.4 (0.2–0.7) μmol/paw, 0.3 (0.1–0.9) μmol/paw and 3.2 (0.9–11.5) μmol/paw, respectively. The antagonists of NMDA (MK801, 1 nmol/paw), AMPA/kainate (DNQX, 1 nmol/paw) or ASIC receptors (amiloride, 100 nmol/paw) failed to reduce the spermine-trigged nociception. However, the TRPV1 antagonists capsazepine or SB366791 (1 nmol/paw) reduced spermine-induced nociception, with inhibition of 81 ± 10 and 68 ± 9%, respectively. The previous desensitization with resiniferatoxin (RTX) largely reduced the spermine-induced nociception and TRPV1 expression in the sciatic nerve, with reductions of 82 ± 9% and 67 ± 11%, respectively. Furthermore, the combination of spermine (100 nmol/paw) and RTX (0.005 fmol/paw), in doses which alone were not capable of inducing nociception, produced nociceptive behaviors. Moreover, different concentrations of spermine (3–300 μM) enhanced the specific binding of [^{3}H]-RTX to TRPV1 receptor. Altogether, polyamines produce spontaneous nociceptive effect through the stimulation of TRPV1, but not of ionotropic glutamate or ASIC receptors.

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1. Introduction

Polyamines putrescine (1,4-butane diamine), spermidine (N-(3-aminopropyl)-1,4-butane diamine) and spermine (N,N0-bis(3-aminopropyl)-1,4-butane diamine) are aliphatic amines initially formed by decarboxylation of ornithine, a reaction catalyzed by the enzyme ornithine decarboxylase (Seiler et al., 1996). Known functions of polyamines include interactions with several biomolecules, especially ion channels (Childs et al., 2003; Rea et al., 2004; Wallace et al., 2003; Williams, 1997).

In fact, various functions of the central nervous system (CNS) are related with polyamine actions on ion channels (Scott et al., 1993). Several *in vitro* and *in vivo* evidences have demonstrated that polyamines can modulate N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) or kainite receptors in CNS (Scott et al., 1993; Williams, 1997). It is also already known that polyvalent cations, such as spermine, are modulators of ASIC receptor activity (Babini et al., 2002). Interestingly, an *in vitro* study has demonstrated that intracellular and extracellular polyamines can modulate the vanilloid receptor TRPV1 activity, a member of the transient receptor potential (TRP) channel family (Ahern et al., 2006). These findings suggest that polyamine could also regulate the function of ion channels in other tissues.

TRPV1, ASIC and glutamatergic NMDA or kainate receptors are expressed by a subset of peripheral pain-sensing neurons (Calixto et al., 2005; Carlton, 2001; Olson et al., 1998). When such receptors are exposed to tissue damaging stimuli, these channels become permeable to Na^+ and Ca^{2+} ions, causing, in turn, neuronal depolarization and recognition as pain stimulus. Sensory neuron firing transmits these pain signals towards the central nervous system, evoking at the same time a variety of local tissue responses (Caterina and Julius, 2001). The peripheral administration of vanilloid, ASIC or glutamate receptor agonists results in nociceptive behavior in mice and rats

* Corresponding author at: Department of Chemistry, Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brazil. Tel.: +55 55 32208053; fax: +55 55 3220 8978.
E-mail address: ferreiraj99@gmail.com (J. Ferreira).

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(Beirith et al., 2002; Ferreira et al., 2004; Meotti et al., 2010; Sakurada et al., 1992; Zhou et al., 1996). On the other hand, peripheral administration of TRPV1, ASIC or NMDA antagonists can attenuate nociception in models of inflammatory pain (Davidson et al., 1997; Meotti et al., 2010; Santos and Calixto, 1997). Of note, the levels of polyamines are increased in tissue and synovial fluid of patients with arthritis (Yukioka et al., 1992), suggesting polyamines could mediate inflammatory pain.

Therefore, the present study aimed to assess the role of peripheral polyamines in nociception *in vivo* and investigate the role of glutamatergic NMDA and AMPA/kainite as well as ASIC and TRPV1 receptors in this action.

2. Methods

2.1. Animals

Male Swiss mice (30–35 g) maintained at 22±2 °C with free access to water and food, under a 12:12 h light:dark cycle, were used. Animals were acclimatized in the laboratory for at least 2 h before testing and were used once throughout the experiments. All experiments were conducted in accordance to the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and "International Guiding Principles for Biomedical Research Involving Animals" (Council of International Organization of Medical Sciences, Geneva, 1985). The number of animals and the nociceptive stimulus were the minimum necessary to demonstrate the consistent effects of drug treatments. This study was approved by the Committee on the Use and Care of Laboratory Animals of our university (no. 23081.012331/2009-81).

2.2. Algogen-induced nociceptive responses

The procedure used was similar to that previously described by Ferreira et al. (2004) and Oliveira et al. (2009). A volume of 20 µl of capsaicin (0.01–3 nmol/paw), glutamate (10,000 nmol/paw), putrescine (1000–10,000 nmol/paw), spermidine (100–2000 nmol/paw) or spermine (100–2000 nmol/paw) was injected subcutaneously (s.c.) under the surface of the right hind paw. Separate groups of animals received s.c. injection of the appropriated vehicle phosphate-buffered saline (PBS) (137 mM NaCl, 27 mM KCl and 10 mM phosphate buffer) or PBS plus ethanol 0.25% for capsaicin. Animals were placed individually in chambers (transparent glass cylinders of 20 cm in diameter) and were adapted for 20 min before treatment. After challenge, mice were observed individually for 5 min and the amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of nociception. The dose and the time of administration of drugs were based on pilot studies.

2.3. Involvement of NMDA, AMPA/kainate, TRPV1, ASIC receptors in spermine-induced nociception

In order to assess the involvement of NMDA, AMPA/kainate or TRPV1 receptors in the nociceptive responses induced by spermine (1000 nmol/paw), animals were co-administered with the selective NMDA receptor antagonist MK 801 (1 nmol/paw), AMPA/kainate receptor antagonist DNQX (1 nmol/paw), acid-sensitive ion channel (ASIC) blocker amiloride (100 nmol/paw), TRPV1 receptor antagonist capsazepine (0.1–1 nmol/paw), the selective TRPV1 receptor antagonist SB366791 (1 nmol/paw) or the TRPV1 agonist resiniferatoxin (RTX; 0.005 pmol/paw). The choice of the dose of antagonists was based on previous data described in literature (Beirith et al., 2002; Ferreira et al., 2004; Ferreira et al., 2005; Oliveira et al., 2009; Meiotti et al., 2010). Control animals received a similar volume of vehicle (20 µl/paw). To confirm the efficacy of the antagonist tested, we tested the effect of MK801 (1 nmol/paw) and DNQX (1 nmol/paw) co-administration with glutamate (10,000 nmol/paw), as well as capsazepine (1 nmol/paw) and SB366791 (1 nmol/paw) co-administration with capsaicin (1 nmol/paw).

2.4. Desensitization of TRPV1 positive fibers in spermine-induced nociception

To further explore the role of TRPV1 positive (TRPV1+) fibers in the nociceptive induced by spermine, the animals were submitted to a systemic desensitization protocol with RTX as previously described by Hsieh et al. (2008). Animals were anesthetized with isoflurane and received systemic administration of RTX (50 µg/kg) or the vehicle alone (0.5% ethanol, 0.5% Tween-80, PBS). After 7 days, animals were submitted to a subcutaneous injection of spermine (1000 nmol/paw, s.c.), capsaicin (1 nmol/paw, used as a positive control) or vehicle (0.5% ethanol, 0.5% Tween-80 and PBS) (20 µl/paw).

2.5. TRPV1 receptor expression following systemic RTX treatment

The effect of the systemic treatment with RTX on the expression of TRPV1 receptors was assessed by Western blot analysis. The assay was carried out as previously described by Andre et al. (2004) with minor modifications. The sciatic nerves were quickly isolated and were homogenized in a lyses buffer containing 10 mM HEPES, pH 7.9, 10 M KCl, 2 M $MgCl_2$, 0.1 M ethylenediamine tetraacetic acid (EDTA), 1 M NaF, 10 µg/ml aprotinin, 10 M β-glycerolphosphate, 1 mM phenylmethanesulphonyl fluoride, 1 M DL-dithiothreitol (DTT) and 2 M of sodium orthovanadate. After centrifugation (3000×*g* for 30 min), the supernatant was collected. The protein content was determined by the method of Bradford (1976), using bovine serum albumin as a standard. Amounts of 30 µg of protein of sciatic nerve were mixed in load buffer (200 mM Tris, 10% glycerol, 2% SDS, 2.75 mM β-mercaptoethanol and 0.04% bromophenol blue) and boiled for 10 min. Proteins were separated in 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride membranes, according to the manufacturer's instructions (Perkin Elmer, USA). The Ponceau staining served as a loading control. After staining the membranes were dried, scanned and quantified. Membranes were processed using a SNAP i.d. system (Millipore, USA), blocked with 1% BSA in TBS-T (0.05% Tween 20 in Tris–borate saline) and then incubated for 10 min, with specific rabbit polyclonal IgG antibodie to anti-TRPV1 (catalog number sc-28759; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:150 in TBS-T. Blots were washed three times with TBS-T followed by incubation with alkaline phosphatase-coupled secondary antibody (1:3000) for 10 min. The protein bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate/p-nitro blue tetrazolium system (BCIP/NBT). Membranes were dried, scanned and quantified with Scion Image PC version of Macintosh compatible NIH image. The TRPV1 western blot had a faint background that was corrected in the image analysis.

2.6. [^{3}H]-RTX binding assay

Binding assays were carried out as previously described by Andre et al. (2004) with minor modifications. To obtain membranes for the binding studies, the spinal cords (a rich source of vanilloid receptors) containing the portion between cervical C1 and lumbar L6 segments of mice were removed and disrupted with the aid of a tissue homogenizer in ice-cold buffer A, pH 7.4, which contained 5 mM KCl, 5.8 mM NaCl, 2 mM $MgCl_2$, 0.75 mM $CaCl_2$, 12 mM glucose, 137 mM sucrose, and 10 mM HEPES. The homogenate was firstly centrifuged for 10 min at 1000×*g* in 4 °C. The low-speed pellets were discarded, and the supernatants were further centrifuged for 30 min at 35,000×*g* in 4 °C. The resulting high-speed pellets, re-suspended in buffer A, were stored at −70 °C until assayed. Binding assays were carried out in duplicate with a final volume of 500 µl, containing buffer

A supplemented with 0.25 mg/ml bovine serum albumin, membrane (100 μg/protein) and 50 pM [^{3}H]-RTX in absence or presence of spermine (3–300 μM). For the measurement of non-specific binding, 100 nm non-radioactive RTX was included in some tubes. Assay mixtures were set up on ice, and the binding reaction was then initiated by transferring the assay tubes to a 37 °C water bath. After a 60-minute incubation period, cooling the mixtures on ice terminated the binding reaction, and then 50 μl of bovine α1-acid glycoprotein (2 mg/ml) was added to each tube (to allow the detection of specific binding). Finally, the bound and free membranes of [^{3}H]-RTX were separated by centrifuging for 15 min at 20,000×*g* in 4 °C. The pellet was quantified using scintillation counting. Specific binding was calculated as the difference between the total and non-specific binding.

2.7. Drugs

The following drugs were used: spermine, spermidine, putrescine, MK801 (5S,10R-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate), DNQX (6,7-dinitroquinoxaline-2,3-dione), glutamate, resiniferatoxin, capsazepine and capsaicin, SB366791 (N-(3-methoxyphenyl)-4-chlorocinnamide) (all from Sigma Chemical Company, St Louis, MO, U.S.A.). The stock solutions of the drugs were prepared in 1 mM phosphate-buffered solution (PBS) from Sigma (USA) pH 7.4 in siliconized plastic tubes, maintained at −20 °C and diluted to the desired concentration just before use. Capsaicin, capsazepine, SB366791 and resinferatoxin stock solutions were prepared in absolute ethanol (90%) plus Tween 80 (10%). For drug administration, the final concentration of ethanol and Tween 80 did not exceed 0.5% and did not cause any detectable effect *per se* (results not shown). In addition, radio-labeled [^{3}H]-resiniferatoxin was purchased from Perkin Elmer (USA).

2.8. Statistical analysis

Results are presented as mean ± s.e.m., except ED_{50} or ID_{50} values (*i.e.*, the dose of agonist necessary to produce 50% of the nociceptive response relative to the maximum effect, or the dose of antagonists necessary to reduce the agonist response by 50% relative to the control value, respectively), which are reported as geometric means accompanied by their respective 95% confidence limits. ED_{50} and ID_{50} values were calculated, using non-linear regression (sigmoidal dose–response curve, variable slope) with the GraphPad Prism software. E_{max} (maximal effect) and I_{max} (maximal inhibition) were calculated based on responses of the control group. The statistical significance between the groups was assessed by means of unpaired Student's *t*-test or one-way ANOVA followed by Dunnett's or Student-Newmann–Keuls' (SNK) test. P-values lower than 0.05 were considered as indicative of significance.

3. Results

3.1. Nociception elicited by peripheral polyamines in mice

The subcutaneously administration of capsaicin (0.01–3 nmol/paw), spermine spermidine or putrescine (100–10,000 nmol/paw) in mice produced short-lasting and spontaneous nociception (Fig. 1). This response started just after the administration and did not last more than 5 min. The calculated mean ED_{50} values (and the 95% confidence limits) for capsaicin, spermine, spermidine or putrescine-induced licking were 0.16 (0.07–0.39) nmol/paw, 0.4 (0.2–0.7) μmol/paw, 0.3 (0.1–0.9) μmol/paw and 3.2 (0.9–11.5) μmol/paw, respectively. The maximal nociceptive response caused by capsaicin, spermine, spermidine and putrescine was 51 ± 4, 70 ± 10, 47 ± 14 and 37 ± 11 s, respectively. Since spermine and spermidine were more potent than putrescine in producing nociception, we have chosen a sub-maximal dose of spermine (1000 nmol/paw) to study some mechanisms involved in polyamine-induced nociception.

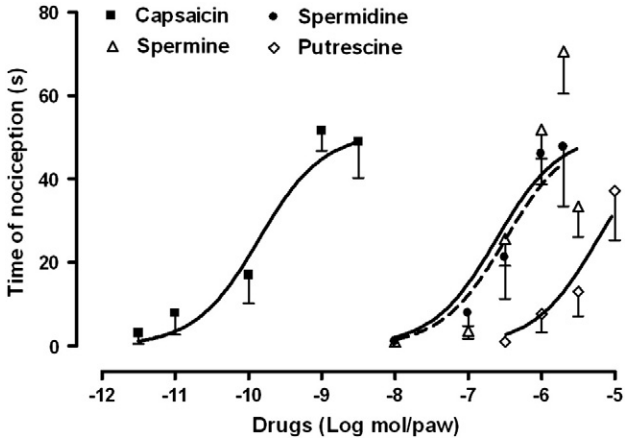

Fig. 1. Dose–response curves for the overt nociception caused by s.c. injection of capsaicin (0.01–3 nmol/paw), spermine (100–2000 nmol/paw), spermidine (100–2000 nmol/paw) or putrescine (1000–10,000 nmol/paw) in mice. The effects of the treatments are expressed as licking time (s). Each bar on the curve represents the mean of 6–10 animals and vertical lines show the s.e.m.

3.2. Lack of participation of NMDA, AMPA/kainate and ASIC receptors in the spermine-induced nociception

The selective NMDA receptor antagonist MK-801 (1 nmol/paw) was not capable of altering spermine-induced nociception (1000 nmol/paw, Fig. 2A). On the other hand, the co-administration of MK-801 (1 nmol/paw) partially, but significantly, reduced the nociception caused by glutamate (10,000 nmol/paw, Fig. 2B) with inhibition of 47 ± 12% compared with the control group. The co-administration of the selective antagonist AMPA/kainate receptor DNQX was also not capable of altering spermine-induced nociception (1000 nmol/paw, Fig. 2C), but partially reduced the nociception caused by glutamate (10,000 nmol/paw, Fig. 2D), with inhibition of 55 ± 6% compared with the control group. Furthermore, we verified that the co-administration of amiloride (100 nmol/paw), an ASIC receptor blocker, was also not able to modify the nociception induced by spermine (1000 nmol/paw; Fig. 3). These results suggest that ionotropic glutamate and ASIC receptors activation did not mediate spermine-induced nociceptive effect.

3.3. Involvement of TRPV1 receptor in the spermine-induced nociception

We have further assessed the possible role of TRPV1 in spermine-induced nociception. The co-administration of the TRPV1 antagonist capsazepine (0.1–1 nmol/paw; s.c.) produced a dose-related and almost complete inhibition of spermine-induced nociception (1000 nmol/paw, Fig. 4A). The calculated mean ID_{50} value for this effect (and its respective 95% confidence limit) was 0.24 (0.03–1.76) nmol/paw and the maximal inhibition was 81 ± 10% compared with the control group. As previously described in literature (Ferreira et al., 2004; Santos and Calixto, 1997) the co-administration of capsazepine (1 nmol/paw; s.c.) also largely reduced the capsaicin-induced nociception (1 nmol/paw, Fig. 4B), with inhibition of 80 ± 6% compared with the control group. To confirm the hypothesis that the spermine-induced nociception is mediated by TRPV1 receptor, the mice received a co-administration of selective TRPV1 receptor antagonist SB366791 (0.3–10 nmol/paw) with spermine. SB366791 also reduced spermine-induced nociception (Fig. 4C), with inhibition of 68 ± 9% compared with the control group and the ID_{50} was 0.6 (0.3–1.4) nmol/paw.

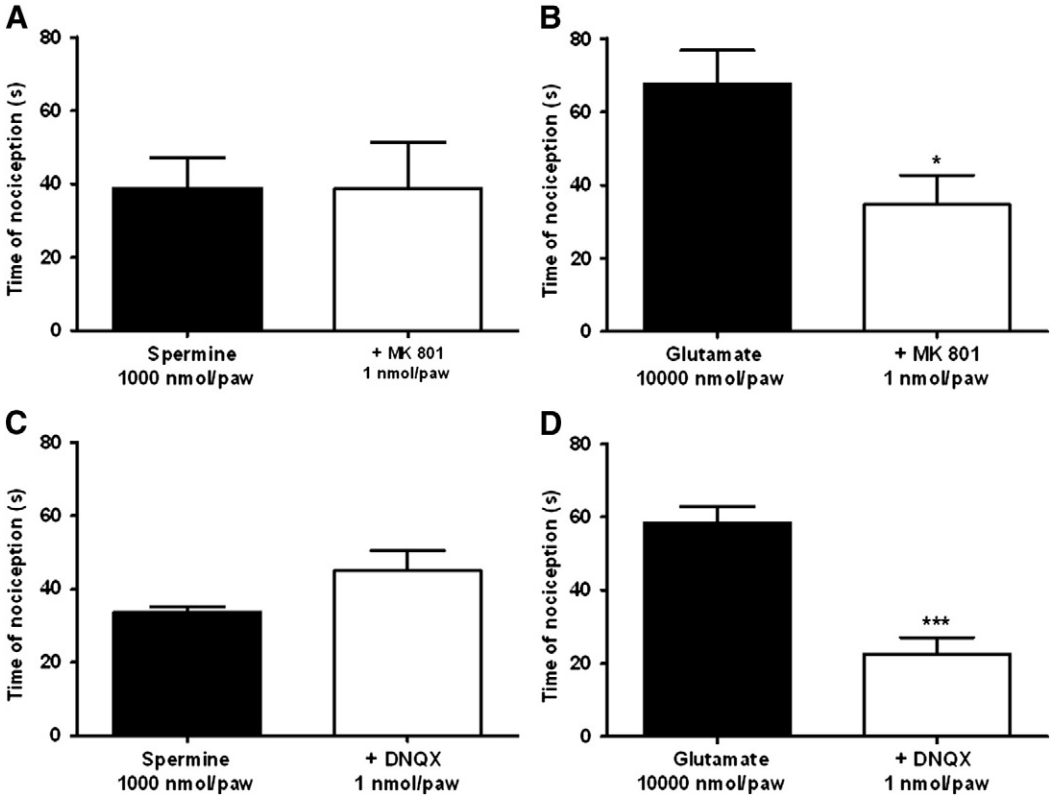

Fig. 2. Effect of s.c. co-treatment with the selective NMDA receptor antagonist MK 801 (1 nmol/paw) or AMPA/kainate receptor antagonist DNQX (1 nmol/paw) on spermine (1000 nmol/paw, A and C) or glutamate-induced nociception (10,000 nmol/paw, B and D). Each column represents the mean ± s.e.m. of 5–6 mice. Asterisks denote the significance levels. *P<0.05, ***P<0.001 compared with glutamate-treated mice (Student's *t* test).

Similarly, the co-administration of SB366791 (1 nmol/paw) reduced the capsaicin-nociception (Fig. 4D), with an inhibition of 72 ± 8% compared with the control group. These data suggest that the activation of TRPV1 receptor is an important mechanism underlying the nociception induced by peripheral injection of spermine in mice.

3.4. Participation of TRPV1 positive fibers in the spermine-induced nociception

To investigate the role of TRPV1 expressing afferent fibers on spermine-induced nociception, animals were submitted to a systemic RTX desensitization protocol. The systemic treatment with RTX significantly reduced the expression of TRPV1 protein in the sciatic nerve 7 days after injection, with an inhibition of 67 ± 11% compared to the control group, confirming a reduction in TRPV1 positive sensory fibers (Fig. 5A). We observed that pre-treatment with RTX (50 μg/kg, s.c., 7 days before) largely reduced the capsaicin (1 nmol/paw) or spermine (1000 nmol/paw)-induced nociception (Fig. 5B), with inhibitions of 92 ± 4 and 82 ± 9% compared with the control group, respectively.

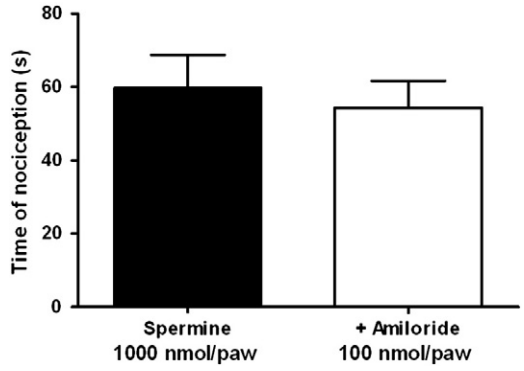

Fig. 3. Effect of co-treatment with the acid-sensitive ion channel (ASIC) blocker amiloride (100 nmol/paw) on spermine (1000 nmol/paw, s.c.)-induced nociception in mice. Each column represents the mean ± s.e.m of 6 mice (Student's *t* test).

3.5. Effect of spermine in resiniferatoxin binding and nociception

To clearly verify the interaction between polyamines with TRPV1 receptor, we performed a specific binding assay of RTX in presence or absence of spermine. It was possible to observe that spermine increases the [^{3}H]-RTX specific binding in the TRPV1 containing membranes, with an IC_{50} value of 54.0 (43.1–67.7) μM and the maximal effect of 76 ± 5% of specific binding enhancement when compared to the control group (Fig. 6A). Again, suggesting that polyamine-induced nociception is mediated by an interaction with TRPV1 receptor, we verified that the co-administration of spermine with RTX, in doses where they alone produced no nociceptive effect, resulted in a significant nociception (Fig. 6B). These results strongly reinforce the idea that polyamines induce nociception by facilitating the activation of TRPV1 receptor.

4. Discussion

Natural polyamines (putrescine, spermidine and spermine) are aliphatic amines containing two (putrescine), three (spermidine) or four (spermine) amine groups (Gerner and Meyskens, 2004). It is well known that they have an important function in the control of the neuronal excitability through direct interaction with different ion

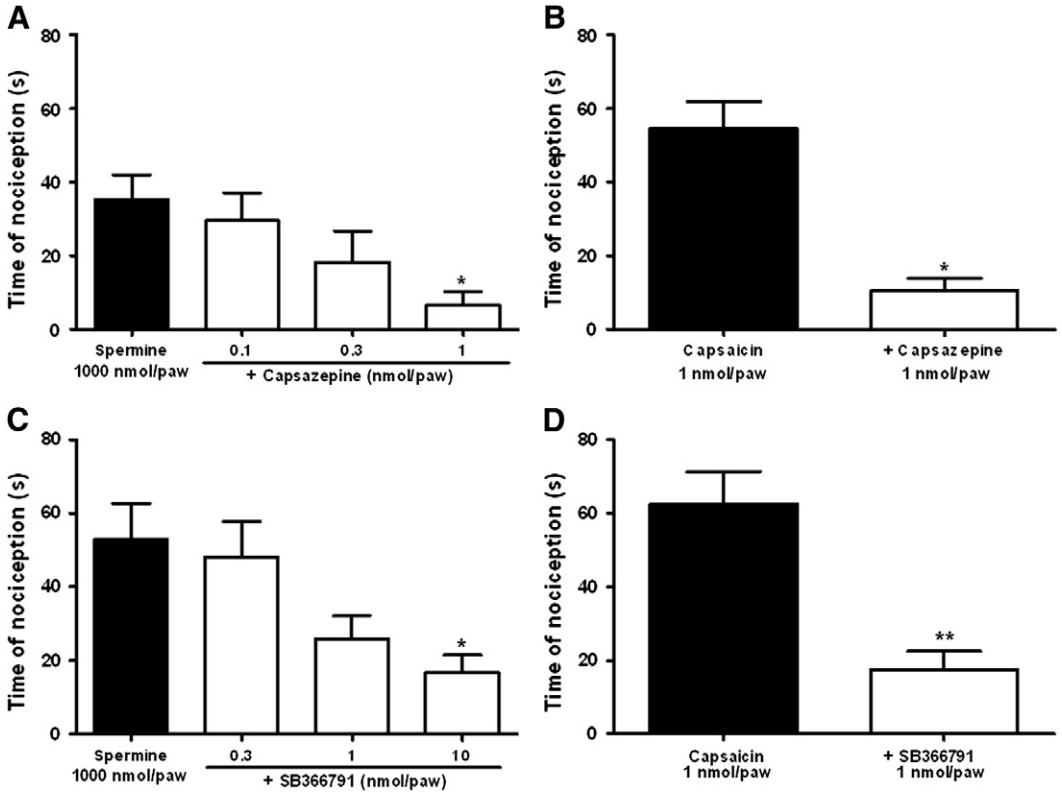

Fig. 4. Effect of s.c. co-treatment with the TRPV1 receptor antagonists capsazepine (0.1–1 nmol/paw) or SB366791 (0.3–10 nmol/paw) on spermine (1000 nmol/paw, A and C) or capsaicin-induced nociception (1 nmol/paw, B and D). Each column represents the mean ± s.e.m. of 4–8 mice. Asterisks denote the significance levels. *P<0.05, **P<0.01 compared with spermine or capsaicin-treated mice (A and C, one-way ANOVA followed by Dunnett's test; B and D, Student's *t* test).

channels, including glutamate receptors NMDA and AMPA/kainate, ASIC and TRPV1 (Ahern et al., 2006; Babini et al., 2002; Williams, 1997). All these ionic channels are important to the development and processing of nociception. Since the levels of polyamines are increased in inflammatory processes (Yukioka et al., 1992) and some drugs acting on polyamines are in clinical trials to treat hyperproliferative disorders (Gerner and Meyskens, 2004; Rivat et al., 2007), the present study could indicate new targets to treat painful conditions. In our study, we have shown that polyamines induced nociception when injected into the mouse paw by stimulation of the TRPV1 receptor present in small diameter afferent fibers (Caterina and Julius, 2001). However, the stimulation of NMDA, AMPA/kainite or ASIC receptors seems to be not important to spermine-induced spontaneous nociception.

We have found that exogenous spermine and spermidine, which are the most charged polyamines, were more potent than putrescine in producing nociception. Our results are in accordance with some data demonstrating that some actions of polyamines in ion channels are charge-dependent (Ahern et al., 2006; Williams, 1997). The pro-nociceptive action of polyamines found here are also in line with previous findings that show that the feeding of rats with a polyamine-deficient diet may produce analgesic action (Estebe et al., 2006; Kergozien et al., 1996; Rivat et al., 2007). Thus, both the restriction of polyamine ingestion and the inhibition of polyamine synthesis could be interesting targets to control painful processes.

It is well known that the modulation of ion channels, including ionotropic glutamate receptors, by polyamines (Williams, 1997). In fact, spermine acts at extracellular sites and at pore in neurons to potentiate the activity of NMDA, AMPA and kainate receptors, respectively. It has been hypothesized that these effects of spermine involve at least two discrete polyamine-binding sites on NMDA receptors (Williams, 1997). In accordance with these data, it has been demonstrated that intrathecal (spinal cord) administration of spermine causes hyperalgesia in rats and pain-related behaviors in mice by modulation of the NMDA receptor (Tan-No et al., 2000; Kolhekar et al., 1994). These results indicate that polyamines in spinal cord can induce nociception through NMDA receptor-stimulation. Furthermore, peripheral administration of NMDA, AMPA/kainite results in painful behavior in rats (Zhou et al., 1996). In contrast with these data, our results demonstrated that the peripheral injection of NMDA receptor antagonist MK 801, in a dose where it is capable of reducing glutamate-induced nociception, failed in altering intraplantar spermine-induced nociception. Thus, we suggest that the peripheral mechanisms involved in spermine-induced nociception are different from the spinal mechanisms. However, the reduction of polyamine-induced nociception by the NMDA antagonists in spinal cord could be indirect since the activation of TRPV1 in spinal cord may release glutamate *in vitro* (Ueda et al., 1993) and produce an NMDA-mediated nociception *in vivo* (Okano et al., 1994). However, further studies must be carried out to elucidate the role of spinal TRPV1 in the nociceptive action of intrathecally-injected polyamines.

Another ion channel that has been implicated in some polyamine action is the acid sensitive ion channel ASIC-1 (Babini et al., 2002). We found that the non-selective ASIC receptor blocker amiloride was not able to reduce the spermine-produced nociception in mice in a dose where it reduced acid-induced nociception (Meotti et al., 2010). Of note, an *in vitro* study has showed that polyvalent cations increased the inactivation of ASIC channel, which could prevent the neuronal depolarization (Babini et al., 2002). Thus, the action of polyamines on ASIC-1 should produce antinociceptive effect instead of a pro-nociceptive action. Moreover, the central nervous system seems to be the major place to the role of ASIC-1 on nociception regulation

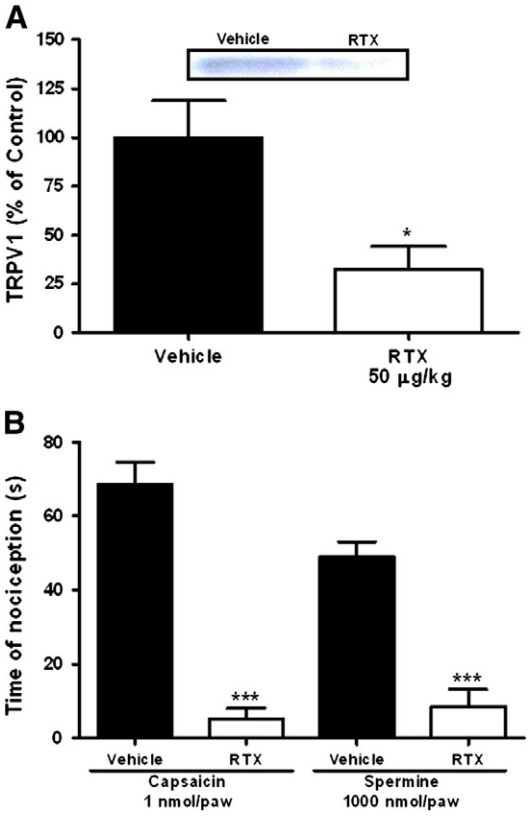

Fig. 5. Western blot analysis showing TRPV1 expression in sciatic nerve (A) after systemic treatment with RTX (50 mg/kg, s.c.) or with vehicle. Effect of RTX desensitization (50 mg/kg, s.c.) on spermine (3 µg/paw, s.c.)-induced nociception in mice (B). Western blot results were represented as arbitrary density unit. Each column represents the mean ± s.e.m of 4–8 mice. Asterisks denote the significance levels. *P<0.05, ***P<0.001 in comparison to control group (A, Student's *t*-test and B, one-way ANOVA followed by Student-Newman–Keuls' test).

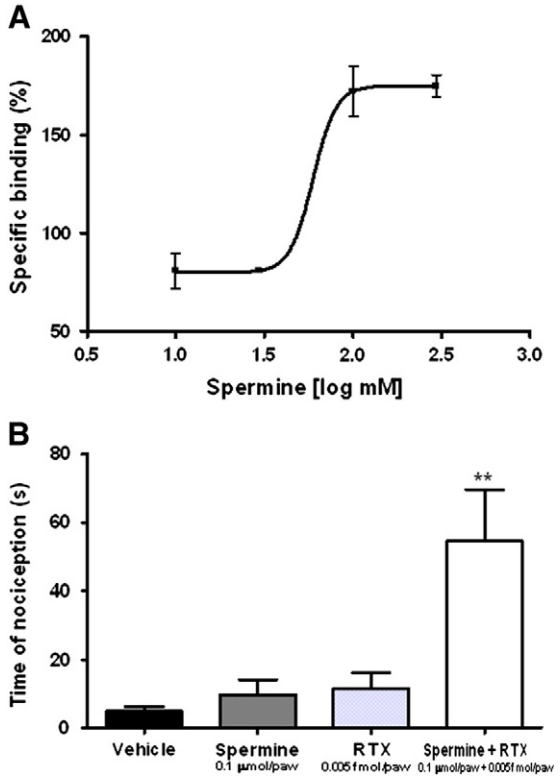

Fig. 6. As specific binding of [^{3}H] resinferatoxin in spinal cord membranes of mice in absence or presence of spermine (3–300 µM) *in vitro* (A). Each point represents the mean ± s.e.m of 3 independent experiments performed in duplicate. Nociceptive effect caused by the s.c. co-injection of low doses of spermine (100 nmol/paw) with resinferatoxin (0.005 nmol/paw) (B). Each column represents the mean ± s.e.m of 4–8 mice. Asterisks denote the significance levels. **P<0.01 compared to control group (B, one-way ANOVA followed by Student-Newman–Keuls' test).

endogenous opioid system (Mazzuca et al., 2007). Thus, the peripheral ASIC-1 receptor seems not to be a target related to polyamine-induced nociception.

Besides ionotropic glutamate and ASIC receptors, polyamines are also capable of directly stimulating TRPV1, as produce ion selective changes in this receptor and resulting in Ca^{2+} influx in sensory neurons (Ahern et al., 2006; Chung et al., 2008). Furthermore, extracellular spermine, spermidine and putrescine directly activate and sensitize TRPV1 in a charge-dependent manner, both in heterologous expression systems and in sensory neurons (Ahern et al., 2006). Here, we have shown that both capsazepine and SB366791 largely reduced spermine-induced nociception with different potencies. Previous studies indeed show that SB366791 is more potent than capsazepine to antagonize TRPV1 receptors from rats and humans (Gunthorpe et al., 2004). This discrepancy may be explained by the fact that the potency and efficacy of TRPV1 antagonists presents species-specific differences (McIntyre et al., 2001). In example, capsazepine fully blocked human and guinea pig TRPV1 receptor to low pH and heat, but only weakly inhibited rat TRPV1 (McIntyre et al., 2001; Savidge et al., 2002). Unfortunately, there are previous studies comparing the effects of SB-366791 and capsazepine on mice TRPV1 receptor. Our data suggest that capsazepine is more potent than SB366791 to antagonize polyamine-induced nociception in mice demonstrating a critical role of TRPV1 in the pain-related behavior caused by peripheral polyamines. However, *in vitro* studies with mice TRPV1 receptor must be carried out to confirm this finding with other TRPV1 agonists.

Furthermore, it has been well established that the treatment of animals with agonists of TRPV1 receptor, such as RTX or capsaicin, is able to produce a selective degeneration of TRPV1 positive fibers, namely sub-types of primary afferent fibers, almost exclusively in peptidergic C fibers, but also in a small number of Aδ fibers (Ferreira et al., 2004; Hsieh et al., 2008; Kobayashi et al., 2005). Our data clearly show that the systemic treatment with RTX significantly reduced the spermine-mediated nociception. This result provides evidence that spermine and RTX activate the same subpopulation of sensory fibers that express TRPV1 receptor and consequently produce nociceptive behavior. The reduction of the TRPV1 protein expression in sciatic nerve confirmed the efficiency of the RTX pre-treatment, which seems to reach to lumbar dorsal root ganglia (Rashid et al., 2003) and consequently to reduce TRPV1 protein in sensory neuron terminals.

Experiments with TRPV1 mutants identified extracellular acidic residues critical for polyamine regulation since the neutralization of aspartate 646 (D646N) abolished direct activation by spermine, whereas neutralization of this same aspartate (D646N) or glutamate 648 (E648A) inhibited spermine-induced sensitization (Ahern et al., 2006). According to this finding, we revealed herein that spermine increased the [^{3}H]-RTX specific binding in the TRPV1 receptor containing membranes. We suggest that the RTX and polyamine binding sites are different, once an increase instead of a competition for the specific binding was observed. In accordance to literature evidence, two residues located in the S2–S3 intracellular loop (Tyr-511, Ser-512) were found to be critical for RTX binding to TRPV1 channels (Jordt and Julius, 2002). Our findings suggest that the polyamine binding on the site in TRPV1 receptor allosterically increases the affinity of the vanilloid site by its ligands. In fact, the combination of RTX with spermine, in doses where they did not produce nociception, resulted in a significant nociceptive response.

Thus, polyamines found in inflamed tissue could increase the binding of endovanilloids in TRPV1 receptor and contribute to pain production.

Of note, increased levels of polyamines are found in tissue and synovial fluid from patients with osteoarthritis or rheumatoid, posttraumatic arthritis and infectious arthritis (Yukioka et al., 1992). In accordance, spermine concentrations up to millimolar levels have been reported in inflammatory conditions (Zhang et al., 2000). Interestingly, the doses of polyamines used in our study to cause spontaneous nociception may be found in inflamed tissues, the concentration of the solution of spermine injected (0.5–150 mM to generate the doses of 0.01–3 μmol/paw) into the mouse paw is in the same range of the spermine concentration in synovial tissue of arthritic patients (about 0.3 mM) (Yukioka et al., 1992). These findings suggest again that polyamine action on TRPV1 could contribute to pain production, especially in inflammatory conditions.

Taken together, these data show that polyamines, probably by virtue of their cationic charge, may increase the activity of TRPV1 in peripheral terminals of sensory neurons and induce nociception. Therefore, polyamine regulation of TRPV1 in peripheral tissues may be relevant to a variety of pathological painful states and represent a target for the treatment of such conditions.

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