Inosine Reduces Pain-Related Behavior in Mice: Involvement of Adenosine A1 and A2A Receptor Subtypes and Protein Kinase C Pathways


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ABSTRACT
Inosine, an endogenous purine, is the first metabolite of adenosine in a reaction catalyzed by adenosine deaminase. This study aimed to investigate the antinociceptive effects of inosine against several models of pain in mice and rats. In mice, inosine given by systemic or central routes inhibited acetic acid-induced nociception. Furthermore, inosine also decreased the late phase of formalin-induced licking and the nociception induced by glutamate. Inosine produced inhibition (for up to 4 h) of mechanical allodynia induced by complete Freund’s adjuvant (CFA) injected into the mouse’s paw. Given chronically for 21 days, inosine reversed the mechanical allodynia caused by CFA. Moreover, inosine also reduced the thermal (cold stimuli) and mechanical allodynia caused by partial sciatic nerve ligation (PSNL) for 4 h; when inosine was chronically administered, it decreased the mechanical allodynia induced by PSNL for 22 days. Antinociception caused by inosine in the acetic acid test was attenuated by treatment of mice with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; a selective adenosine A1 receptor antagonist), 8-phenyltheophylline (8-PT; a nonselective adenosine A1 receptor antagonist), and 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-yl-amino]ethyl}phenol (ZM241385; a selective adenosine A2A receptor antagonist). In rats, inosine inhibited the mechanical and heat hyperalgesia induced by bradykinin and phorbol 12-myristate 13-acetate, without affecting similar responses caused by prostaglandin E2 or forskolin. These results indicate that inosine induces antinociceptive, antiallodynic, and antihyperalgesic effects in rodents. The precise mechanisms through which inosine produces antinociception are currently under investigation, but involvement of adenosine A1 and A2A receptors and blockade of the protein kinase C pathway seem to largely account for inosine’s antinociceptive effect.

The purinergic system, including ATP and its metabolite adenosine, plays a relevant physiological role in the control of pain at the peripheral and central levels (Sawynok, 1998). Adenosine is converted to inosine by the action of the enzyme adenosine deaminase (Sawynok and Liu, 2003). Inosine is an endogenous nucleoside with anti-inflammatory effects, and it can be considered a natural trigger of adenosine receptors, which are widely distributed through organs and cell types (Hasko et al., 2000; Gomez and Sitkovsky, 2003). There are four known subtypes of adenosine receptors (A1, A2A, A2B, and A3), and each one contains its own pharmacological profile, tissue distribution, and coupling effectors (Fredholm et al., 2001; Sawynok and Liu, 2003). Inosine inhibits the release of proinflammatory cytokines and chemokines in activated macrophages (Hasko et al.,

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ABBREVIATIONS: CFA, complete Freund’s adjuvant; ANOVA, analysis of variance; PSNL, partial sciatic nerve ligation; PMA, phorbol 12-myristate 13-acetate; PKA, protein kinase A; PKC, protein kinase C; PGF2α, prostaglandin E2; DMSO, dimethyl sulfoxide; CGS 21680, 3-[4-[2-[9-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid.
Prolonged inflammation or nerve injury in humans often leads to a sensory nociceptor sensitization called hyperalgesia or allodynia. Neuropathic pain is a kind of chronic pain that can persist for days, months, or even years after nerve injury. It has been demonstrated that neuropathic pain is attenuated by adenosine in humans (Belfrage et al., 1995). Furthermore, adenosine analogs and adenosine kinase inhibitors produce effects in animal models of neuropathic pain (Sawynok, 1998). Several studies have extensively reported the effect of adenosine against nociception (Sawynok, 1998; Sawynok and Liu, 2003); however, no evidence has been shown about the antinociceptive effect of inosine. For this reason, we aimed to investigate the antinociceptive effects of inosine against writhing, formalin, and glutamate tests. In addition, this study addressed the effects of inosine in neuropathic and inflammatory chronic pain in mice and hyperalgesic pain in rats. Finally, we evaluated the role played by the adenosine receptors by using selective adenosine A₁, A₂A, A₂B, and A₃ receptor agonists and antagonists in mice.

Materials and Methods

Animals. Experiments were conducted with male Swiss mice (25–35 g) and male Wistar rats (250–350 g) obtained from the Universidade Federal de Santa Catarina’s animal facility. Animals were housed at 22 ± 2°C under a 12-h light/12-h dark cycle (lights on at 6:00 AM) and had access to food and water ad libitum. 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 Committee for Animal Research of the Universidade Federal de Santa Catarina and 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 (Zimmermann, 1983). The numbers of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Abdominal Constriction Response Caused by Intraperitoneal Injection of Acetic Acid. Abdominal constriction is a contraction of the abdominal muscle together with a stretching of the hind limbs in response to an intraperitoneal injection of 0.6% acetic acid at the time of the test (Koster et al., 1959). Mice were pretreated with inosine intraperitoneally (0.1–100 mg/kg) or orally (10–300 mg/kg) 30 and 60 min before the irritant injection, respectively. Other groups of animals were treated with inosine intracerebroventricularly (0.1–10 μg/site) or intrathecally (0.01–10 μg/site) 15 min before the irritant injection. In these studies, control mice received an equal volume of vehicle or saline. After the challenge, mice were individually placed into glass cylinders 20 cm in diameter, and abdominal constrictions were counted cumulatively over a period of 20 min. Antinociceptive activity was expressed as the reduction in the number of abdominal constrictions compared with those of the control group. In addition, we investigated the time course of the antinociceptive effect of inosine given intraperitoneally (10 mg/kg) or orally (100 mg/kg) 0.5, 1, 2, 4, 6, 8, and 12 h before acetic acid administration. Control mice received an equal volume of vehicle and were observed at the same time intervals.

Formalin-Induced Nociception. The procedure used was essentially the same as that described previously (Hunskaar et al., 1985) with minor modifications. Animals received 20 μl of a 2.5% formalin solution (0.92% formaldehyde) made up in saline, injected intraplantarly in the ventral surface of the right hind paw. Animals were observed from 0 to 5 min (neurogenic phase) and 15 to 30 min (inflammatory phase). Animals received inosine (0.1–100 mg/kg i.p.) 30 min beforehand. Control animals received vehicle (10 ml/kg i.p.). After the intraplantar injection of formalin, animals were immediately placed in a glass cylinder 20 cm in diameter. The time they spent licking the injected paw was recorded with a chronometer and considered as indicative of nociception.

Glutamate-Induced Nociception. To provide more direct evidence concerning the interaction of inosine with the glutamatergic system, we investigated whether inosine was able to antagonize glutamate-induced licking of the mouse paw. The procedure used was similar to that described previously (Beirith et al., 2002). A volume of 20 μl of glutamate (10 μmol/paw prepared in saline) was injected intraplantarly in the ventral surface of the right hind paw. Animals were observed individually for 15 min after glutamate injection. The time they spent licking the injected paw was recorded with a chronometer and was considered as indicative of nociception. Animals were treated with inosine (0.01–10 mg/kg i.p.) 30 min before glutamate injection. Control animals received a similar volume of vehicle intraperitoneally (10 ml/kg).

Measurement of Locomotor Activity. The open-field test was used to rule out the possibility that the antinociceptive action of inosine could be related to nonspecific disturbances of the locomotor activity of the animals. The ambulatory behavior was assessed in an open-field test as described previously (Rodrigues et al., 2002). The apparatus consisted of a wooden box measuring 40 × 60 × 50 cm. The floor of the arena was divided into 16 squares. The number of squares crossed with all paws (crossing) was counted in a 6-min session. Mice were treated with inosine (1–100 mg/kg i.p.) or vehicle (10 ml/kg i.p.) 30 min beforehand. The apparatus was cleaned with a solution of 10% ethanol between tests to hide animal clues.

Partial Sciatic Nerve Ligation. Mice were anesthetized with an intraperitoneal injection of 10 mg/kg ketamine and 80 mg/kg xylazine. A partial ligation of the sciatic nerve was performed by tying the distal 1/3 to 1/2 of the dorsal portion of the sciatic nerve, according to the procedure in mice described by Malmberg and Basbaum (1998). In sham-operated mice, sciatic nerves were exposed without ligation. The wound was closed and covered with iodine solution. The animals were divided into four groups. The sham-operated mice received inosine (10 or 30 mg/kg i.p.) or vehicle (10 ml/kg i.p.), and sham-operated animals received only vehicle (10 ml/kg i.p.) 7 days after surgery. The mechanical (von Frey monofilaments) and thermal (cold stimuli) allodynia responses were recorded before (baseline) and after (0, 0.5, 1, 2, 4, 8, 12, and 24 h) treatment to verify the time-course effect of inosine in inhibiting the allodynia responses. To investigate the effects of the long-term treatment on mechanical allodynia, inosine (10 or 30 mg/kg i.p.) was administered to mice twice a day (every 12 h). The allodynia response was evaluated 1 h after the first treatment (time with maximal inhibition observed in the acute treatment). The repeated treatment extended from the 7th to the 22nd day after partial sciatic nerve ligation (PSNL), and then it was interrupted for 3 days. Next, the treatment was reinitiated to assess the development of possible tolerance effect of inosine.

Measurement of Thermal (Cold Stimuli) Alldynia. For assessment of allodynia by cold stimulus in mice with PSNL, the Cold/Hot Plate Analgesia Meter (Columbus Instruments, Columbus, Ohio) was used. Mice were placed on the cold plate (2°C) and handled at the time until the shake of the hind paw was recorded. The cutoff latency for this test was 30 s. Animals were habituated 7 days before surgery,
and the presurgery baseline was measured 1 day before the partial ligation of the sciatic nerve (Osikowicz et al., 2008).

**Measurement of Mechanical Allodynia.** The mechanical allodynia was measured as described previously (Bortolanza et al., 2002). The withdrawal response frequency to 10 applications of 0.4 g of von Frey filaments (Stoelting Co., Wood Dale, IL) were recorded as the nociceptive percentile value. Mice were further acclimatized in individual clear boxes (9 × 7 × 11 cm) on an elevated wire mesh platform to allow access to the ventral surface of the hind paws. The frequency of withdrawal was determined before and after PSNL.

**Complete Freund’s Adjuvant-Induced Chronic Inflammatory Pain.** Mice were injected with 20 μl of 30% complete Freund’s adjuvant (CFA) (Mycobacterium tuberculosis; Sigma-Alrich, St. Louis, MO) (intraplantarly) as described by Ferreira et al. (2001) with minor modifications. The sham group received 20 μl of phosphate buffer saline in the ipsilateral paw. CFA produced significant hind paw swelling and hyperalgesia. To assess the effects of the acute treatment of inosine against CFA-induced chronic inflammatory pain, animals received inosine (30 mg/kg i.p.) 24 h after CFA intraplantar injection. Development of mechanical allodynia was evaluated at 0, 0.5, 1, 2, 4, and 24 h after treatment to verify the time-course effect of inosine in inhibiting the allodynia responses (Bortolanza et al., 2002). To investigate the effects of the long-term treatment on mechanical allodynia, inosine (30 mg/kg i.p.) was administered in mice once a day. The allodynia response was evaluated 4 h after the treatment (time with maximal inhibition observed in the acute treatment). The repeated treatment extended from the 1st to the 22nd day after CFA injection, and it was interrupted for 4 days. Next, the treatment was reintroduced to assess the development of a possible tolerance effect of inosine.

**Bradykinin-, Phorbol 12-Myristate 13-Acetate-, Prostaglandin E₂-, or Forskolin-Induced Mechanical and Thermal Hyperalgesia Response.** The possible antihyperalgesic effect of inosine was evaluated as described previously (Randall and Selitto, 1957; Hargreaves et al., 1988; Otuki et al., 2005). The animals were pretreated intraperitoneally with inosine (10 mg/kg) or vehicle (10 ml/kg; control group) 30 min before injection of 100 μl of phorbol 12-myristate 13-acetate (PMA; 0.1 nmol/paw), bradykinin (BK; 3 nmol/paw), prostaglandin E₂ (PGE₂; 10 nmol/paw), forskolin (1μmol/paw), or saline only into the right hind paw. The hyperalgesia was evaluated 30 min later. When bradykinin was used, animals were pretreated with captopril (5 mg/kg s.c.), an angiotensin-converting enzyme inhibitor, 1 h before the experiments to prevent its degradation (Otuki et al., 2005; Parada et al., 2005).

**Measurement of Mechanical and Thermal (Hot Stimuli) Hyperalgesia.** The nociceptive threshold (of squeak response or paw withdrawal) was assessed by applying increasing pressure or temperature to the dorsal site of inflamed or control rat paws, using an analogs meter and a thermal withdraw apparatus (both Ugo Basile, Comero, Italy) as described previously (Randall and Selitto, 1957; Hargreaves et al., 1988) with minor modifications. The weight on the analogs meter ranged from 0 to 750 g, and the threshold was expressed as load (g) tolerated. For the thermal threshold, rats were habituated to the environment for approximately 30 min before testing. Withdrawal latencies were measured automatically with photocell light. A cutoff time was set at 20 s to avoid tissue damage. Light intensity was preset to obtain baseline latency between 10 and 15 s.

**Involvement of Adenosine A₁, A₂A, A₂B, and A₃ Receptors.** To investigate the role played by the adenosine A₁, A₂A, A₂B, and A₃ receptors in the antinociceptive effect of inosine in the acetic acid test, mice were pretreated with vehicle (10 ml/kg i.p.), 1,3-dipropyl-8-cyclopentlylamphetamine (DPCPX, a selective adenosine A₁ receptor antagonist) (0.1 mg/kg i.p.), 8-phenyltheophylline (8-PT; a nonselective adenosine A₁ receptor antagonist) (3 mg/kg i.p.), 4-[2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-ylamino]ethyl]phenol (ZM241385; a selective adenosine A₂B receptor antagonist) (3 mg/kg i.p.), alloxazine (a selective adenosine A₂B receptor antagonist; 3 mg/kg i.p.), and 2-phenoxoy-6-(cyclohexylamino)purine hemioxalate (MRS-3777; a selective adenosine A₃ receptor antagonist) (5 mg/kg i.p.). After 20 min, every group received an injection of inosine (10 mg/kg i.p.), N⁶-cyclohexyladenosine (CHA; a selective adenosine A₁ receptor agonist) (0.1 mg/kg i.p.), N⁶-[2-(3,5-dimethoxyphenyl)-2(methylphenyl)ethyl]adenosine (DPMA; a nonselective adenosine A₂ receptor agonist) (1 mg/kg i.p.), 2-hexyn-1-yl-N⁶-cyclohexyladenosine (HEMADO; a selective adenosine A₃ receptor agonist) (1 mg/kg i.p.), or vehicle (10 ml/kg i.p.), and 30 min later they were subjected to the acetic acid test. The doses of the drugs used were based on literature data (Garcia Soriano et al., 2001; Bastia et al., 2002, Schmidt et al., 2009) and previous results from our laboratory.

**Drugs.** Acetic acid, formalin, dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany), inosine, l-glutamic acid hydrochloride, CHA, bradykinin, CFA, captopril, PGE₂, (Sigma-Alrich), xylazine, ketamine (Vetbrands International, São Paulo, Brazil), morphine sulfate (Cristalia Ind., São Paulo, Brazil), PMA, alloxazine, HEMADO, and MRS-3777 (Tocris Bioscience, Ellisiaville, MO) were dissolved in saline (0.9% NaCl). DPMA, 8-PT, forskolin (Sigma-Alrich), DPCPX, and 4-[2-[7-amino-2-(2-furyl)]1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl]phenol (ZM241385) (Tocris Bioscience) were dissolved in saline with 5% DMSO. The final concentration of DMSO did not exceed 5% and did not cause any effect per se.

**Statistical Analysis.** The results are presented as mean ± S.E.M., except for ID₅₀ values (i.e., the dose of inosine reducing the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their 95% confidence limits. The ID₅₀ values were determined by nonlinear regression analysis from individual experiments using Prism 4.0 (GraphPad Software Inc., San Diego, CA). The statistical significance of differences between groups was detected by one-way ANOVA followed by Newman-Keuls test or two-way ANOVA followed by Bonferroni test. P values less than 0.05 were considered indicative of significance.

**Results**

**Abdominal Constriction Response Caused by Intraperitoneal Injection of Acetic Acid.** The results depicted in Fig. 1A show that inosine, given intraperitoneally 30 min before testing, produced dose-related inhibition of the acetic acid-induced abdominal contractions in mice with a mean ID₅₀ value (and 95% confidence limits) of 5.78 (2.66–12.53) mg/kg, and the inhibition observed was 80 ± 8% for the dose of 10 mg/kg. Furthermore, given orally 60 min before testing, inosine also produced dose-related inhibition of the acetic acid-induced abdominal contractions in mice with a mean ID₅₀ value of 101.00 (71.83–142.10) mg/kg and inhibitions of 70 ± 5% for the dose of 300 mg/kg (Fig. 1B). Hence, inosine was approximately 20-fold more potent in preventing the nociception caused by acetic acid when it was given intraperitoneally than when given orally. A time-course analysis of the antinociceptive effect of inosine given intraperitoneally or orally is shown in Fig. 1, A and B, insets. Inosine produced marked antinociception as early as 30 min after intraperitoneal administration, an action that remained significant up to 6 h after administration. When inosine was administered orally, its effect was significant until 2 h after administration. Thus, the administration of inosine intraperitoneally (30 min before testing) was chosen for all further studies with independent groups of animals.

The results presented in Fig. 1, C and D show that inosine, administered intrathecally or intracerebroventricularly 15 min before testing, produced dose-related
inhibition of the acetic acid-induced writhing in mice with mean ID$_{50}$ values of 0.44 (0.16–1.20) and 2.68 (0.64–11.14) µg/site and inhibition of 71 ± 5 and 56 ± 6%, respectively, for the dose of 10 µg/site. Hence, inosine was approximately 6-fold more potent in preventing the nociception caused by acetic acid when it was given intrathecially than when given intracerebroventricularly.

Formalin-Induced Nociception. The treatment of animals with inosine 30 min before the test did not decrease the time that the mice spent licking or biting the injected paw in the early phase of the formalin test (Fig. 2A). However, in the late phase of the formalin test, inosine significantly diminished the nociceptive response compared with mice treated with vehicle (p < 0.001). The mean ID$_{50}$ value was 2.59 (1.15–5.88) mg/kg, and the percentage of inhibition was 91 ± 3% (Fig. 2B).

Glutamate-Induced Nociception. The results presented in Fig. 2C show that inosine, given intraperitoneally, caused a significant and dose-related inhibition of the glutamate-induced nociception compared with mice treated with vehicle (p < 0.001) with a mean ID$_{50}$ value of 0.72 (0.22–2.36) mg/kg and inhibition of 57 ± 6% for the dose of 1 mg/kg.

Measurement of Locomotor Activity. Inosine treatment (1–100 mg/kg i.p.) did not alter the locomotor activity of mice in the open-field test compared with animals that received vehicle (control group) until 8 h after treatment. The means ± S.E.M. of crossing numbers 30 min after administration were 188.0 ± 7.1, 188.9 ± 5.4, 191.6 ± 7.8, and 175.9 ± 6.6 for the control group and groups receiving 1, 10, and 100 mg/kg inosine, respectively (data not shown).

Neuropathic Pain-Like Behavior After Partial Sciatic Nerve Ligation. The PSNL, a neuropathic pain model,
reduced the basal threshold of cold and mechanical allodynia in the ipsilateral hind paw compared with the nonoperated group (p < 0.05). Figure 3, A and B shows that acute treatment with inosine (30 mg/kg i.p.) reduced both cold (81 ± 8%) and mechanical (54 ± 7%) allodynia responses produced by PSNL 30 min after treatment. Its action remained significant up to 4 h after its administration in both tests.

The long-term treatment of animals with inosine (30 mg/kg i.p.) twice a day markedly decreased the mechanical allodynia response produced by PSNL 30 min after treatment. Its action remained significant up to 4 h after its administration in both tests.

CFA-Induced Chronic Inflammatory Pain. Results depicted in Fig. 4A show that inosine given intraperitoneally (30 min before testing) produced a significant and time-dependent inhibition of the mechanical allodynia induced by intraplantar injection of CFA in the right paw with maximal inhibition of 82 ± 12%. Inosine produced pronounced antinociception as early as 30 min after intraperitoneal administration and remained significant up to 4 h. When inosine was chronically administered (once a day) for 21 days, it also significantly reduced the mechanical allodynia induced by CFA (Fig. 4B). This effect was evident until day 15 of treatment (inhibition of 87 ± 11% on the 15th day). When the treatment was interrupted for 4 days, the mechanical allodynia was re-established. When treatment was restarted on the 20th day, inosine once again significantly reduced the mechanical allodynia.

Bradykinin-, Phorbol 12-Myristate 13-Acetate-, Prostaglandin E2-, or Forskolin-Induced Mechanical and Thermal Hyperalgesia. Results of Fig. 5 show that intraplantar administration of bradykinin (3 nmol/paw), PMA (0.1 nmol/paw), PGE2 (10 nmol/paw), and forskolin (1 μmol/paw) significantly increased (p < 0.01) the sensitivity to mechanical stimuli (hyperalgesia) in rats when assessed by the Randall-Selitto test. Furthermore, the treatment of rats with inosine (10 mg/kg i.p., 30 min before testing) reversed

Fig. 2. Effects of inosine administration on the formalin- and glutamate-induced nociception in mice. A and B, the total time spent licking the hind paw was measured in the early phase (0–5 min; A) and late phase (15–30 min; B) after intraplantar injection of formalin. Each column represents the mean of eight animals, and the vertical lines indicate the S.E.M. C, effects of inosine administered intraperitoneally on glutamate-induced nociception in mice. The total time spent licking the hind paw was measured 0 to 15 min after intraplantar injection of glutamate. Column C indicates the control values (animals treated with the vehicle), and the asterisks denote significance levels compared with control groups (one-way ANOVA followed by Newman-Keuls test; **, P < 0.01 and ***, P < 0.001).

Fig. 3. Effects of inosine on partial sciatic nerve ligation-induced neuropathic pain. A, effect of treatment with inosine (30 mg/kg i.p.) given 7 days after the partial sciatic nerve injury on the thermal hyperalgesia test (cold plate) in mice. The nociceptive responses of animals were measured from 0 to 8 h after administration of inosine. B, effect of acute administration of inosine (10 and 30 mg/kg i.p.) on mechanical hyperalgesia induced by PSNL in ipsilateral paws in mice. The nociceptive responses of animals were measured from 0 to 24 h after administration of inosine. C, in chronic analysis the nociceptive responses of animals were measured from the 7th to the 22nd day. Each point represents the mean of eight animals. Two-way ANOVA followed by Bonferroni test, significantly different from the sham-operated mouse group; ***, P < 0.001; **, P < 0.01; and *, P < 0.05. B, baseline withdrawal threshold.

Neuropathic pain
Inflammatory chronic pain

Fig. 4. Effects of inosine on CFA-induced nociception. A, effect of acute administration of inosine (10 mg/kg i.p.) on mechanical hyperalgesia induced by CFA injection in the ipsilateral paws of mice. The nociceptive responses of animals were measured from 0 to 24 h after administration of inosine. B, in chronic analysis the nociceptive responses of animals were measured until the 22nd day. Each point represents the mean of eight animals. Two-way ANOVA followed by Bonferroni test, significantly different from the sham-operated mouse group; ***, P < 0.001; **, P < 0.01; and *, P < 0.05. B, baseline withdrawal threshold. In some cases, the error lines are hidden within the symbols.

Hyperalgesic pain

Fig. 5. Effects of inosine against mechanical and thermal hyperalgesia in rats. Effect of intraperitoneal administration of inosine (10 mg/kg, i.p.) against PMA- or BK-, prostaglandin E2-, or forskolin-induced mechanical (A) and thermal (B) hyperalgesia in rats. Each column represents the mean of eight rats, and the error bars indicate the S.E.M. The symbols denote significance levels: #, p < 0.05 compared with PMA or BK versus inosine plus PMA or BK (one-way ANOVA followed by Newman-Keuls test); ***, p < 0.001; **, p < 0.01; and *, p < 0.05 compared with corresponding control values (animals pretreated with vehicle alone).

Analysis of Mechanism of Action of Inosine. The results depicted in Fig. 6 show that previous treatment of mice with DPCPX (a selective adenosine A1 receptor antagonist; 0.1 mg/kg i.p.) and 8-PT (a nonselective adenosine A1 receptor antagonist; 3 mg/kg i.p.), given 20 min before testing, significantly reversed the antinociception caused by CHA (a selective adenosine A1 receptor agonist; 0.1 mg/kg i.p.), HEMADO (a selective adenosine A2 receptor agonist; 1 mg/kg i.p.), and inosine (10 mg/kg i.p.) when assessed in the acetic acid-induced abdominal constrictions in mice. Moreover, Fig. 6 also shows that the pretreatment of animals with ZM241385 (a selective adenosine A2A receptor antagonist; 3 mg/kg i.p.) completely reversed the antinociception caused by DPMA (a nonselective adenosine A2A receptor agonist; 1 mg/kg i.p.) and partially reversed the antinociception caused by inosine (10 mg/kg i.p.) against acetic acid-induced pain. Moreover, the pretreatments of animals with MRS-3777 (a selective adenosine A3 receptor antag-
Purine nucleosides such as adenosine and its primary metabolite inosine participate as extracellular signaling molecules, influencing synaptic transmission and modulating the activity of the nervous system (Cunha, 2001). In addition, it is recognized that adenosine and its analogs participate in several systems and have a significant role in the perception of pain at both peripheral and central sites in a variety of pain models in human and animals, including acute, neuropathic, and inflammatory pain (Sawynok, 1998; Sawynok and Liu, 2003). Results reported here indicate, to our knowledge for the first time, that systemic (intraperitoneal or oral) or central (intrathecal or intracerebroventricular) administration of inosine produced marked and dose-related antinociception when assessed in acetic acid-induced visceral nociception. Moreover, our results also show that inosine decreased the nociception during the second phase of the formalin test and reduced the nociceptive response caused by glutamate, which involves peripheral, spinal, and supraspinal sites (Beirith et al., 2002). Together, these results suggest that the antinociceptive action of inosine in acetic acid-, formalin-, and glutamate-induced pain could be caused by the inhibition of the release of proinflammatory mediators, such as prostaglandins, glutamate, histamine, and others. In addition, it has been shown that adenosine inhibits the release of cytokines and chemokines in activated macrophages and increases the production of the anti-inflammatory cytokine interleukin-10 (Haskó et al., 2000; García Soriano et al., 2001; Németh et al., 2005).

At present, few drugs are effective in treating chronic pain, and thus the search for new mechanisms that could be applied in chronic pain therapy has been essential. We also demonstrated that acute treatments of animals with inosine are effective in preventing mechanical and thermal allodynia caused by PSNL in mice. Chronically, inosine also presents antinociceptive action in mechanical allodynia induced by PSNL. The nociceptive response induced by PSNL occurs because of the release of multiple inflammatory and nociceptive mediators, producing an increased long-lasting discharge of primary sensory fibers that modifies neuronal, neuro-glial, and neuro-immune cell phenotype and function in the central nervous system and induces hyperalgesia and allodynia (Ji and Woolf, 2001). The purine nucleoside adenosine plays an important role in pain modulation and might play an important role in neuropathic pain (Sawynok, 1998; Dunwiddie and Masino, 2001). Adenosine alleviates spontaneous pain in patients with neuropathic pain (Belfrage et al., 1995). Thus, our findings demonstrate that inosine, similar to adenosine, reduces neuropathic pain, and its effect could depend on the activation of $A_1$ and $A_{2A}$ receptors. In addition, we injected CFA into the paw of mice, and inosine decreased the nociception induced by CFA. Our results show that acute or chronic treatment of animals with inosine was effective in preventing the persistent mechanical allodynia caused by CFA. This effect probably involves the inhibition of protein kinases and anti-inflammatory properties of this nucleoside.

Another interesting finding of the present study was the demonstration, also for the first time, that inosine, given intraperitoneally, was able to reverse PMA- and bradykinin-induced mechanical and thermal hyperalgesia in the rat paw. Several inflammatory mediators produce nociception by peripheral and spinal sensory fibers sensitization through protein kinase activation, including protein kinase C (PKC), protein kinase A (PKA), and mitogen-activated kinases (Scholz and Woolf, 2002). Results of the present study also strongly suggest the involvement of protein kinase C, but not protein kinase A, in the antinociception caused by inosine. This notion was derived from data showing that inosine inhibited the overt mechanical and thermal hyperalgesia caused by intraplantar PMA injection (a PKC activator). Other evidence that supports this view involved the results demonstrating that inosine suppressed the mechanical and thermal hyperalgesia induced by bradykinin. Some studies propose that in nociception bradykinin binds to the $B_2$ receptor, causing a direct activation of PKC (Ferreira et al., 2004), suggesting that PKC isoforms are involved in signal transduction of the $B_2$ receptor. In contrast, inosine did not reduce nociception induced by prostaglandin $E_2$ or forskolin, both indirect activators of protein kinase A (Parada et al., 2005).

In the present study, the involvement of $A_1$ and $A_{2A}$ receptors in the antinociceptive action of inosine in the writhing test is clearly demonstrated. Our results show that pretreatment of animals with DPCPX, 8-PT, or ZM241385 at doses that did not cause any effect by themselves significantly...
reversed the antinociception caused by inosine. In addition, we observed that the antagonism of the adenosine A1 and A2A receptor by DPCPX or 8-PT and ZM241385 significantly inhibited the antinociception induced by adenosine A1 and A2A receptor agonists CHA and DPMA, respectively. In addition, HEMADO (an adenosine A3 receptor agonist) partially inhibited the nociception induced by acetic acid. In this case, HEMADO could be acting on the A2 receptor, because DPCPX and 8-PT reversed this effect. Thus, our finding is in agreement with those reported by other authors, indicating that adenosine A1 receptor agonists produce a pronounced antinociception (Bastia et al., 2002; Sawynok and Liu, 2003).

Nevertheless, the role of the receptor A2A in nociception has been intensely debated. Moreover, it has been demonstrated that A2A receptor antagonists showed consistent antinociceptive activity (Doak and Sawynok, 1995), and hypoalgesia in mice lacking the adenosine A2A receptor was observed (Ledent et al., 1997). In the formalin test, CGS 21680 [3-4-2-[6-amino-9-(2R,5R,4S,5S)-5-(ethyl-carbamoyl)-3,4-dihydroxy-oxolan-2-yl][purin-2-yl]amino] ethyl]phenyl]propanoic acid; agonist A2A has been shown to produce both antinociceptive (Borghi et al., 2002) and pronociceptive actions in rats (Doak and Sawynok, 1995). However, some authors have shown that the activation of the adenosine A2A receptor has an antinociceptive role against the chemical (writhing test) and thermal model of pain beyond inflammatory and neuropathic pain tests (Lee and Yaksh, 1996; Poon and Sawynok, 1998; Pechlivanova and Georgiev, 2002; Loram et al., 2009). The antinociceptive effect of adenosine A2A receptor activation could be at least in part caused by potassium channel activation (Regaya et al., 2004). In addition, DPMA, an A2 receptor agonist, produces antinociceptive action in the writhing test (Pechlivanova and Georgiev, 2002).

We demonstrated that DPCPX and 8-PT did not reverse the effect caused by DPMA, clearly demonstrating that the A2A receptor is involved in antinociception induced by inosine. Our result disagrees with other studies showing that the antinociceptive effect of A2A agonists occurs only in very high doses, when there is also activation of the A1 receptor (Ferré et al., 2007). Thus, involvement of the A2A receptor could depend on the intensity and modality of the stimulus. Additional studies are required to determine precisely the role of the adenosine A2A receptor in distinct locales (i.e., peripheral, spinal, or supraspinal) and under different situations. Although our results are in agreement with a study by Haskó et al. (2000) that shows that inosine inhibits inflammatory cytokines production and this effect was partially reversed by blockade of adenosine A1 and A2 receptors, we cannot affirm unequivocally that inosine binds to A1 and A2A adenosine receptors. Another explanation for the antinociceptive effect caused by inosine would be an increase of adenosine’s production after inosine administration, caused by a change on purinergic metabolism caused by inhibition of enzymes adenosine deaminase or ectoadenosine kinase. This action could lead to a greater supply of adenosine that activates their respective receptors. However, the effect caused by inosine is an interesting avenue of investigation and merits further study.

Our results also demonstrated that the A3B receptor did not participate in the antinociceptive effect caused by inosine, because alloxazine (an antagonist A2B receptor) did not change the effect induced by inosine. In pain tests, A3 adenosine receptor knockout mice showed a decreased sensitivity to some painful stimuli (Fedorova et al., 2003) or did not differ compared with wild-type mice in their mechanical and heat sensitivity (Wu et al., 2002). Furthermore, an injection of A3 agonists produced transient pain-like behaviors and paw edema (Sawynok et al., 1999). Our study shows that the adenosine A3 receptor is not involved in the antinociceptive effect of inosine in the writhing test, because MRS-3777, a selective antagonist A3 receptor, was not capable of reducing the antinociception caused by inosine.

In conclusion, the present results indicate that inosine produces a pronounced effect against the nociception induced by acetic acid, formalin, and glutamate. In addition, inosine reduced the mechanical and thermal (cold stimuli) allodynia induced by FSNL and mechanical allodynia induced by CFA in mice. Moreover, inosine reduced mechanical and thermal (heat stimuli) hyperalgesia caused by algogenic substances (bradykinin and PMA) in rats. The mechanisms through which inosine exerts its action are currently under investigation, but an involvement of adenosine A1 and A2A receptors and blockade of the PKC pathway seem largely to contribute to the antinociceptive effect of inosine.

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References


