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The antinociceptive effects of the systemic adenosine A1 receptor agonist CPA in the absence and in the presence of spinal cord sensitization

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Abstract

Adenosine A1 receptor agonists are effective antinociceptive agents in neuropathic and inflammatory pain, though they appear to be weak analgesics in acute nociception. Important discrepancies are observed on the effectiveness and potency of adenosine analogues when comparing different studies, probably due to the use of different ligands, models of antinociception, routes of administration and types of sensitization. We studied the systemic antinociceptive effects of the adenosine A1 receptor agonist *N*6-cyclopentyladenosine (CPA) in spinal cord neuronal responses from adult male rats in acute nociception and in sensitization due to arthritis and neuropathy. The experiments showed that CPA was effective in the three experimental conditions, with a similar potency in reducing responses to noxious mechanical stimulation (ID50s: 20 ± 1.2 μg/kg in acute nociception, 18 ± 1.1 μg/kg in arthritis, 17.4 ± 2 μg/kg in neuropathy). The phenomenon of wind-up was also dose-dependently reduced by CPA in the three experimental situations although the main action was seen in arthritis. Depression of blood pressure by CPA was not dose-dependent. We conclude that systemic CPA is a potent and effective analgesic in sensitization due to arthritis and neuropathy but also in acute nociception. The effect is independent of the cardiovascular activity and is centrally mediated since wind-up was inhibited.

Keywords: Pain; Analgesia; Inflammation; Neuropathy; Single motor unit; Wind-up

1. Introduction

Adenosine A1 receptor agonists are effective antinociceptive agents in neuropathic pain, as shown in several studies using different nerve injury models (Sawynok, 1998; De Vry et al., 2004), as well as in inflammatory pain (Sawynok, 1998; Dickenson et al., 2000; Sawynok and Liu, 2003). However, the effectiveness and potency of adenosine analogues are very disparate and important differences are observed when comparing studies. In addition, it seems that adenosine and its derivatives only produce weak analgesic actions in rodent models of acute nociception (Keil and DeLander, 1992), although there are some studies indicating the opposite (for review see Sawynok and Liu, 2003). There is also controversy on the location of the antinociceptive effect of adenosine agonists. Many studies show that adenosine A1 receptor agonists are involved in spinal cord-mediated antinociception (Karlsten et al., 1991; Reeve and Dickenson, 1995; Lee and Yaksh, 1996; Sawynok and Liu, 2003), especially in situations of sensitization. In fact, adenosine A1 receptors are highly concentrated on dorsal horn neurons (Geiger et al., 1984; Choca et al., 1988), and the spinal application of A1 receptor agonists induces a potent antinociceptive effect (Lee and Yaksh, 1996; Sawynok, 1998), including an important inhibition of the wind-up phenomenon (Reeve and Dickenson, 1995; Suzuki et al., 2000). Adenosine A1 receptors, however, are present in most areas of the brain (see Ribeiro et al., 2003 for review) and supraspinal inhibitory activity of the adenosine A1 system has been suggested by electroencephalography (Fulga and Stone, 1998) and other techniques (Phillis et al., 1975; Uchimura and North, 1991). Supporting a supraspinal antinociceptive activity of adenosine is a study made in our lab (Ramos-Zepeda et al., 2004) in which we observed that the adenosine A1 receptor selective agonist *N*6-cyclopentyladenosine (CPA) was a very effective antinociceptive drug after systemic administration in adult rats with an intact spinal cord, but not in animals with the spinal cord transected. This

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observation indicated that, in our hands, the main action of systemic CPA was located supraspinally in adult rats or, at least, that the presence of supraspinal modulation is essential for the antinociceptive actions mediated by the adenosine A1 system in the adult animal.

Controversial results on the antinociceptive and antihyperalgesic activity resulting from the activation of adenosine A1 receptors might derive from the differences in the ligands utilized, in the experimental conditions and in the routes of administration. The aim of the present study was to investigate the influence of the spinal cord sensitization on the antinociceptive activity of the adenosine A1 receptor agonist CPA, and the duration of its effect, in the absence and in the presence of monoarthritis and neuropathy using the same experimental protocol of stimulation and the same technique of evaluation of antinociception. In order to avoid differences due to the lack of supraspinal receptor activation, the antinociceptive activity of CPA was evaluated after systemic administration. Spinal cord nociceptive activity was elicited by noxious mechanical stimulation and by high intensity repetitive electrical stimulation that triggers the centrally mediated phenomenon of wind-up. An additional aim was to examine whether adenosine receptor activation is involved in the modulation of the wind-up phenomenon in the three experimental conditions, in order to discriminate a peripheral from a central action (Herrero et al., 2000).

2. Methods

The antinociceptive activity of the A1 receptor selective agonist *N*6-cyclopentyladenosine (CPA) was studied in electrophysiological experiments performed on 19 adult male Wistar rats weighing 225-380 g, that were divided into three groups: i) normal animals ($n=6$), ii) animals with carrageenan-induced monoarthritis ($n=6$) and iii) animals with mononeuropathy ($n=7$). Monoarthritis was induced 16 h before the experiment under halothane anesthesia (5% in oxygen for induction and 2% for maintenance) by the injection of 50 μl of carrageenan λ (Sigma, 10 mg/ml, in distilled water) into the right knee cavity. The degree of articular inflammation produced by carrageenan was assessed by comparing the knee perimeter before and 16 h after the induction of inflammation. Mononeuropathy was induced seven days before the experiment under the same halothane regime following the partial sciatic nerve ligation technique (Seltzer et al., 1990), a ligation that induces mechanical allodynia, heat-evoked hyperalgesia, and spontaneous pain, which are present for up to 7 months. The development of hyperalgesia induced by the sciatic nerve ligation was assessed by behavioral experiments, studying withdrawal reflex responses evoked by mechanical and thermal stimulation. The frequency of withdrawal reflex responses to mechanical stimulation was studied by applying von Frey filaments. A response was considered as positive when a withdrawal of the paw due to the application of the stimulus was observed. The methods were adapted from those described by Gilchrist et al. (1996) and have been described in detail elsewhere (Mazario et al., 2001). The rats were placed on a raised wire mesh grid under plastic chambers. Six von Frey filaments (60, 80, 100, 200, 300 and 500 mN) were applied ten times for approximately 1 s to the plantar surface of each hind paw in an ascending series. The frequency of paw withdrawal was calculated for each filament. Thermal hyperalgesia was assessed by measuring paw withdrawal latencies to 55 °C radiant heat generated by an algesimeter (Ugo Basile plantar test, Hargreaves et al., 1988). Animals were placed in a clear plastic chamber and were allowed to accommodate to the testing apparatus for 5 min. Two consecutive thermal stimuli were applied to each of the paws with an interval of 2–3 min between tests and the maximum cutoff time was set to 17 s to avoid tissue damage. Control tests were established previous to the nerve ligation. Further tests were made at 1, 4 and 7 days after the induction of neuropathy.

The preparatory surgery for electrophysiological experiments was performed under halothane anesthesia (5% in oxygen for induction and 2% for maintenance) and consisted of the cannulation of the trachea, two superficial branches of the jugular veins (for the administration of anesthesia and drugs) and one carotid artery. Details of methods have been described previously (Herrero and Headley, 1991; Solano and Herrero, 1997). Halothane was discontinued after surgery and the anesthesia was maintained with α-chloralose (50 mg/kg for induction and 25 mg/kg/h, by perfusion pump, for maintenance in a rate of 1 ml/h to assure a correct animal hydration). Core temperature was maintained at 37±0.5 °C by means of feedback controlled blanket. Blood pressure was monitored continuously and systolic levels were always above 100 mm Hg before the administration of the drug. In all cases the preparation was left to rest for at least 1 h after the surgery before any drug was tested.

The recording of withdrawal reflexes as single motor units (SMUs) has been used to test the analgesic activity of different drugs and has been described in detail several times (Herrero and Headley, 1991; Romero-Sandoval et al., 2003; Gaitan et al., 2003; Ramos-Zepeda et al., 2004). Briefly, SMU activity was recorded from hind limb muscles by means of a bipolar Teflon-coated tungsten electrode. Activity was elicited in 3 min cycles consisting of noxious mechanical stimulation (10 s, 200 mN above threshold over 14 mm^2) and one train of percutaneous electrical stimuli (2 ms pulse width, 1 Hz and twice the threshold intensity for the recruitment of C-fibers) applied to the most sensitive area of the cutaneous receptive field of the unit (Protocol of stimulation in Fig. 1). Mechanical stimulation was performed by a computer-controlled pincher device. The threshold force was considered as the minimum force required to trigger a sustained nociceptive reflex over the period of 10 s of stimulation. Only units with a steady firing rate were selected for experiments. Electrical stimulation was used to study the phenomenon of wind-up (see Herrero et al., 2000 for review). Data from electrical stimulation were analyzed by counting the responses evoked between 150 and 650 ms after each pulse, considered as C-fiber mediated inputs. At the end of the experiments the animals were killed with an overdose of sodium pentobarbital (Euta-Lender,

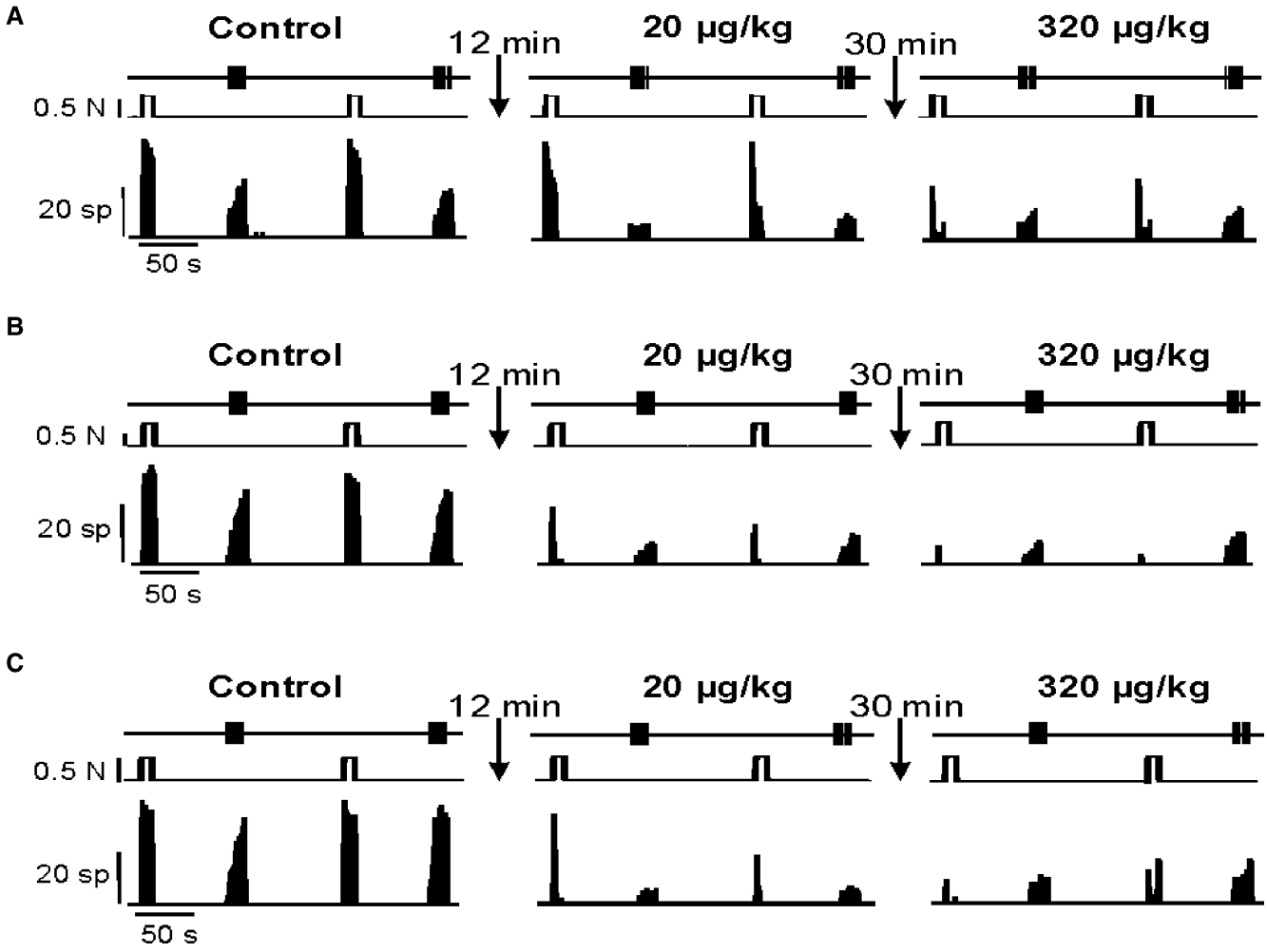

Fig. 1. Original recordings of three different single motor units previous to and after the administration of iv. cumulative doses of CPA in normal (A), arthritic (B) and neuropathic animals (C). Units were activated in 3 min cycles by 10 s of noxious mechanical stimulation and 16 electrical pulses (2 ms pulse width, 1 Hz and twice the threshold intensity for the recruitment of C-fibers). CPA was administered iv. in log2 cumulative doses every two cycles of stimulation (6 min) from 10 to 320 µg/kg. The administration of CPA dose-dependently reduced the nociceptive responses in the three experimental conditions.

Normon). All experiments in this study were undertaken in accordance with European Union legislation (European Community Council Directive of 24 November 1986; 86/609/EEC) regarding the uses of animals for experimental protocols and all efforts were made to reduce the number of animals used.

CPA (Sigma) was dissolved in DMSO (Sigma) 0.5 µg/µl and diluted in saline. The drugs were prepared everyday, immediately before the administration, and were injected in cumulative log2 regime every 2 cycles of stimulation (6 min) in a total and constant volume of 0.3 ml. The initial dose used was 10 µg/kg and the highest dose was 320 µg/kg (Fig. 1 illustrates the protocol of stimulation and administration of drugs). The administration of each dose of CPA was made very slowly, for a minimum time of 3 min so as to minimize the effect on blood pressure. The effect of the highest cumulative dose was studied for a minimum of 30 min. Data are presented as percentage of control, control being the average of the three responses previous to the administration of the drug (mean ± s.e.m.). Drug effect was assessed with the one-way analysis of variance (ANOVA) with post hoc Dunnett's test.

3. Results

The mean forces used for mechanical stimulation in the three experimental groups were 1.1 ± 0.1 N in normal animals, 0.92 ± 0.1 N in arthritic animals and 1.2 ± 0.1 N in animals with neuropathy. The mean intensities of electrical stimulation were 3.6 ± 1.1, 2.3 ± 0.4 and 3.4 ± 0.8 mA, respectively. No significant differences in the intensity of stimulation were observed in the three experimental groups. Mean control number of responses elicited by noxious mechanical stimulation was also very similar and no significantly different: 356 ± 42 spikes in the normal situation, 326 ± 46 spikes in arthritis and 312 ± 14 spikes in neuropathy. The injection of carrageenan induced a significant increase of the knee circumference (64 ± 1.1 to 75 ± 1.3 mm; $P < 0.01$; Fig. 2). Likewise, the induction of neuropathy enhanced the number of responses to mechanical stimulation ($P < 0.01$; Fig. 2) and reduced the latency to noxious thermal stimulation ($P < 0.01$; Fig. 2).

The administration of iv. cumulative doses of CPA reduced dose-dependently the responses of SMUs to noxious mechanical stimulation in the three experimental groups (Figs. 1 and

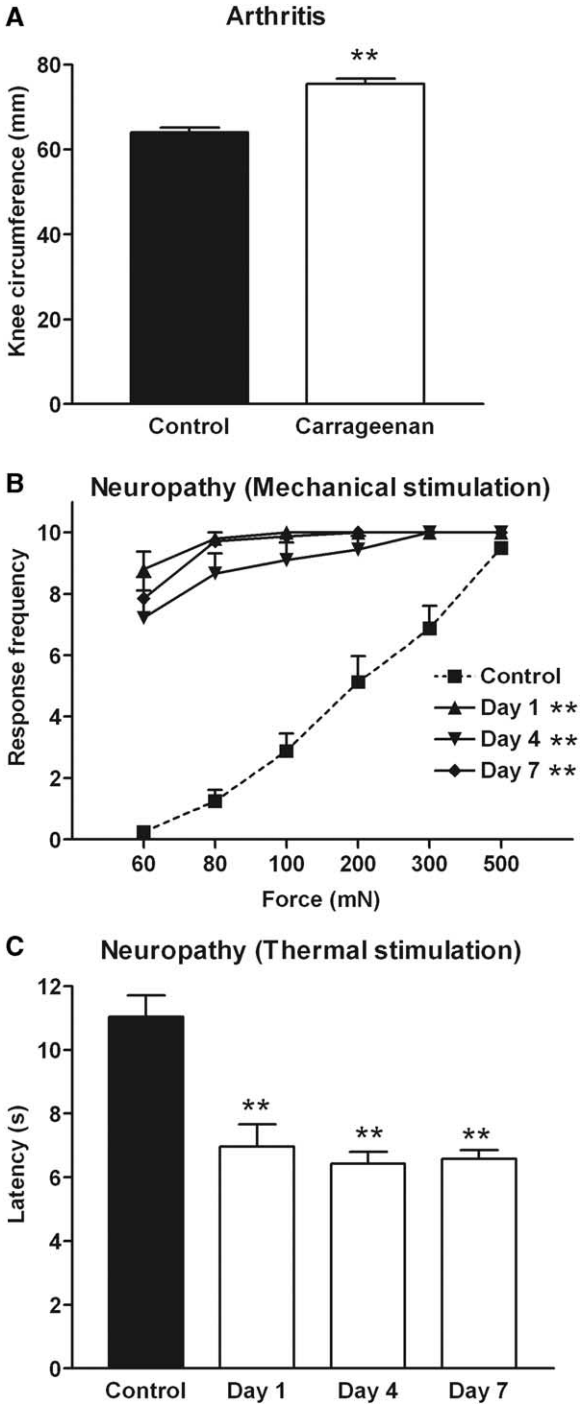

Fig. 2. Induction of sensitization. (A) The administration of 50 μl of carrageenan in the knee joint induced an evident inflammation. The circumference of the knee increased significantly 16 h after the injection of carrageenan. Neuropathy was induced seven days before the experiment following the partial sciatic nerve ligation technique. (B) Mechanical hyperalgesia was studied by applying a series of von Frey filaments previous to and at days 1, 4 and 7 after the nerve ligation. (C) Thermal hyperalgesia was assessed by measuring paw withdrawal latencies to 55 °C radiant heat using a similar timing. An intense mechanical and thermal hyperalgesia was observed in all tests made after the induction of neuropathy (*$P<0.05$, **$P<0.01$, comparison vs. control response with the one-way ANOVA, with the post hoc Dunnett's test).

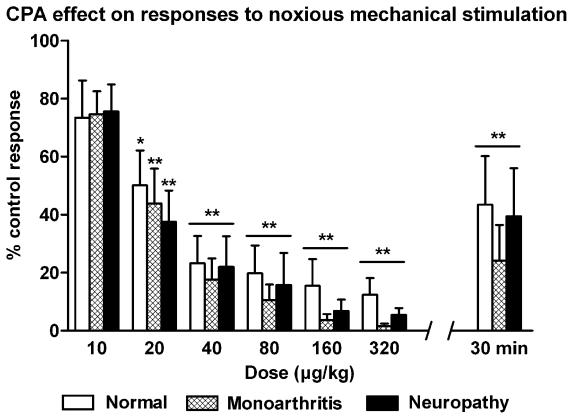

Fig. 3. Pooled data of the antinociceptive effect of CPA in responses to noxious mechanical stimulation. The iv. administration of CPA induced a dose-dependent inhibition of responses to noxious mechanical stimulation in the three experimental groups. The calculated ID50s were of 20±1.2 mg/kg in the normal situation, 18±1.1 mg/kg in arthritis and 17.4±2 mg/kg in neuropathy. The effect was still significant 30 min after the administration of CPA. Statistical significance and layout as for Fig. 2.

3). The calculated ID50s were of 20±1.2 μg/kg in the normal situation, 18±1.1 μg/kg in arthritis and 17.4±2 μg/kg in neuropathy. No significant differences were observed, both in the potency and effectiveness of CPA, in the three groups. The effect of CPA was still significant 30 min after administration in the three situations (Fig. 3). All the units studied showed a progressive increment of the number of spikes with repetitive electrical stimulation (wind-up). The phenomenon of wind-up was significantly and dose-dependently reduced by CPA in the three experimental situations but with a different intensity. Only a partial inhibition of wind-up (53%±14% of control, $P<0.01$) was observed in the normal situation. In arthritis, CPA induced a full inhibition (16%±5% of control, $P<0.01$) of wind-up, whereas in neuropathy, the level of reduction was somehow in between the other two situations (38%±18% of control, $P<0.01$) (Fig. 4). Depression of wind-up was still significant 30 min after the injection of CPA in the normal and arthritic situation but not in neuropathy (Fig. 3). Despite the slow administration of CPA, a strong depression of blood pressure was observed in all cases: 24%±4% of control in the normal situation, 26%±3% of control in arthritis and 26%±4% of control in neuropathy with the highest dose used. The effect of CPA on blood pressure was not dose-dependent and the depression of blood pressure observed with the highest cumulative dose was not significantly different of that observed with the lowest dose used (41%±6% of control in the normal situation, 40%±6% of control in arthritis and 43%±6% of control in neuropathy). A recovery of blood pressure above 50% was always observed 30 min after the administration of CPA.

4. Discussion

The main observation made in the present experiments is the intense and dose-dependent inhibition of nociceptive responses caused by the systemic administration of the adenosine A1 receptor agonist, CPA, in the acute nociceptive situation and in

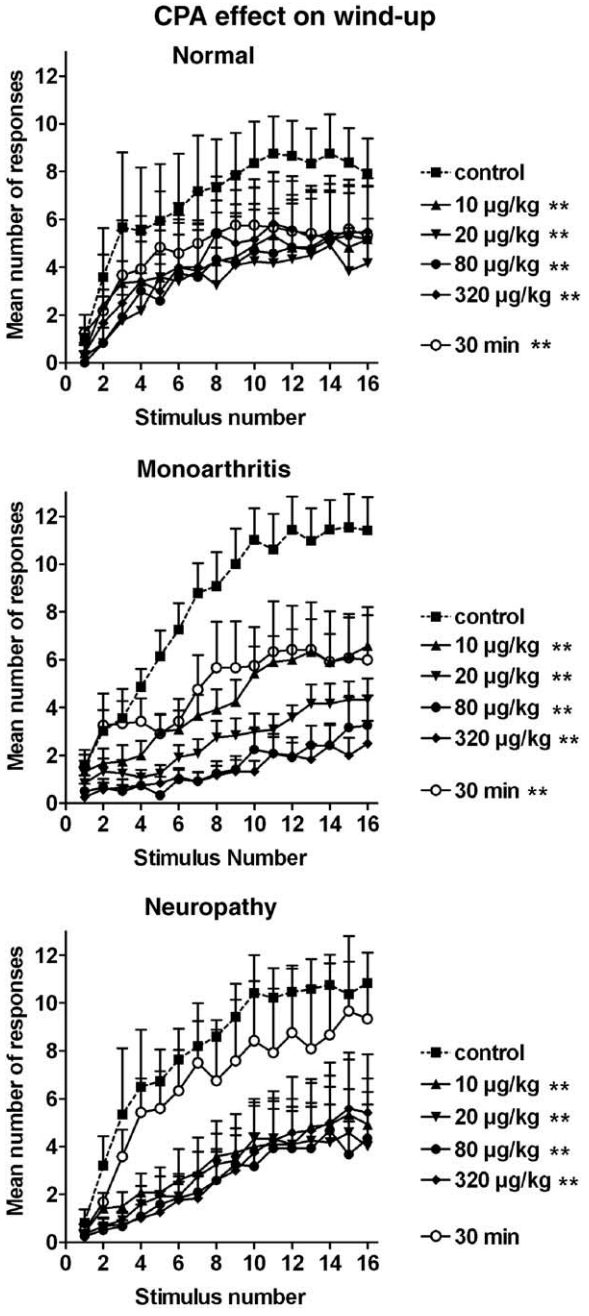

Fig. 4. Effect of CPA on wind-up responses. The administration of CPA dose-dependently reduced the wind-up phenomenon in the three experimental conditions. The effect was partial in acute nociception (normal, 53%±14% of control) and more intense in arthritis (16%±5% of control) and neuropathy (38%±18% of control). Statistical significance and layout as for Fig. 2 (Only some doses are shown for clarity).

situations of sensitization due to articular inflammation and neuropathy. The administration of carrageenan induced an intense inflammation of the knee joint, indicating the development of inflammation and, therefore, sensitization. The level of inflammation was similar to that observed in previous studies and was associated to an intense hyperalgesia (Herrero and Cervero, 1996a,b). Behavioral experiments, in turn, showed an intense level of hyperalgesia to both mechanical and thermal stimulation, similar to that observed in previous experiments (Seltzer et al., 1990), indicating the presence of neuropathy-induced sensitization. The recording of nociceptive withdrawal reflexes as single motor units is a technique that allows the recording of direct spinal cord neuronal responses with a minimum of preparatory surgery, and that has been shown to be useful in this type of studies (Herrero and Headley, 1991; Herrero and Solano, 1999; Mazario et al., 1999, 2001; Romero-Sandoval et al., 2002). In addition, the current protocol allowed us to test for analgesic effects of an adenosine A1 receptor agonist on similar experimental conditions and on different situations of spinal cord sensitization, as well as distinguishing effects mediated at peripheral nociceptor endings from those occurring centrally. Natural stimulation of the skin is transduced by nociceptors, and thus analgesic agents acting either in the periphery or centrally will reduce nociceptive responses. Electrical stimulation, however, bypasses nociceptors and directly activates afferent axons, thus analgesics acting on nociceptors will have no effect on responses to electrical stimulation.

In the present study, the effectiveness and potency of CPA were very similar in the three experimental conditions showing that the activation of adenosine A1 receptors triggers an important antinociception, which is independent of the presence or absence, intensity or type of central sensitization. The effect was also of long duration, since only a low recovery was observed 30 min after the administration and was effective in responses to both mechanical and electrical stimulation. A depressive action of adenosine analogues on wind-up has been previously described in situations of central sensitization after direct spinal cord (Reeve and Dickenson, 1995; Suzuki et al., 2000) or systemic administration (Ramos-Zepeda et al., 2004). To our knowledge, this is the first study to show that CPA depresses wind-up after systemic administration in acute nociception as well as in sensitization due to arthritis and neuropathy. An action at central sites is supported by the strong effect exerted on the wind-up phenomenon. In this phenomenon, repetitive electrical stimulation induces a progressive increase of nociceptive responses from spinal cord neurons (Herrero et al., 2000, and references within) and is mediated by NMDA (Davies and Lodge, 1987; Dickenson and Sullivan, 1987) and NK1 receptors (De Felipe et al., 1998). A reduction of wind-up implies an inhibitory action of the circuitry involved in its generation, which is located in the central nervous system, at spinal cord level (see Herrero et al., 2000 for further discussion), although a modulation of the system by higher levels in the CNS is also possible (Herrero and Cervero, 1996b). The effect of CPA, therefore, takes place presumably at central sites rather than in the periphery.

The antinociceptive activity of CPA was accompanied by an intense depression of blood pressure, as reported previously. In fact, it is well known that adenosine is a potent modulator of cardiovascular function and produces hypotension and bradycardia when administered systemically (Barraco et al., 1987; Evoniuk et al., 1987). These effects have been thought to be mediated at adenosine receptors localized both in central areas of the nervous system and in the periphery. However, a recent study shows that cardiovascular actions produced by CPA and other adenosine analogues are preferentially mediated by

peripheral adenosine A1 receptors, with little or no contribution of central adenosine A1 receptors (Schindler et al., 2005). This finding supports that the antinociceptive activity observed in our experiments is independent of the cardiovascular depression. In addition, the depression of blood pressure observed in the present study was not dose-dependent and the level of hypotension observed was similar with all the doses studied and totally disparate to the reduction of nociceptive activity. This also supports a lack of relationship between the inhibition of nociception and the depression of cardiovascular function.

In conclusion, our results show that CPA is a potent and effective analgesic agent in situations of sensitization due to arthritis and neuropathy but also in the acute nociceptive situation after intravenous administration. The effect is independent of the cardiovascular activity and is mediated at central sites since wind-up was greatly inhibited.

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