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Spinal vs. supraspinal antinociceptive activity of the adenosine A_1 receptor agonist cyclopentyl-adenosine in rats with inflammation

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Abstract

The adenosine A_1 receptor is involved in spinal cord antinociception. As its role at supraspinal sites is not well known, we studied the systemic effects of its agonist *N*-cyclopentyl-adenosine (CPA) in single motor units from adult-spinalized, intact and sham-spinalized rats. CPA was not effective after spinalization, but it was very effective in intact animals (ID50: 92±1.3 μg/kg, noxious pinch) and over 10-fold more potent in sham-spinalized animals (ID50 of 8.3±1 μg/kg). Wind-up was also inhibited by CPA. We also studied the effect of CPA in the immature spinal cord preparation, where CPA dose-dependently inhibited responses to low (IC50s: 9±0.7 and 7.7±1.3 nM) and high intensity stimulation (IC50s: 4.9±0.5 and 12.1±2 nM). We conclude that the integrity of the spinal cord is crucial for the antinociceptive activity of systemic CPA in adult rats but not in immature rats, not yet influenced by a completely developed supraspinal control.

Keywords: Pain; Wind-up; Single motor unit; Spinal cord; Adenosine; Inflammation

1. Introduction

Adenosine is considered an inhibitory modulator of neuronal activity and is involved in a wide number of regulatory actions in the nervous system, including roles in the control of cardiovascular function, sleep, neuroprotection, locomotion and nociception (Dunwiddie and Masino, 2001). Adenosine A_1 receptor agonists have been implicated 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, as adenosine itself only produces weak analgesic actions in rodent models of acute nociception (Keil and Delander, 1992). In fact, adenosine A_1 receptors are highly concentrated on dorsal horn neurons (Geiger et al., 1984; Choca et al., 1988) and the spinal application of adenosine A_1 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). In addition, the adenosine A_1 receptor agonist cyclopentyl-adenosine (CPA) depresses miniature excitatory postsynaptic currents in substantia gelatinosa neurons of adult rat spinal cord slices (Lao et al., 2001). All these studies support an antinociceptive effect of adenosine A_1 receptors within the spinal cord and, as a result, the antinociceptive activity of adenosine has mainly been associated with actions on the spinal cord and at peripheral sites (Sawynok 1998; Sawynok and Liu, 2003). In fact, the antinociceptive action of spinally administered adenosine A_1 receptor agonists has been shown to be mediated by both pre- and postsynaptic adenosine A_1 receptors (Li and Perl, 1994). The activation of presynaptic adenosine A_1 receptors reduces neurotransmitter release from primary afferent terminals due to a $G_{i/o}$ protein-mediated inhibition of voltage gated Ca^{2+} channels (Santicioli et al., 1993; Lao et al., 2001). The activation of postsynaptic adenosine A_1 receptors also diminishes synaptic transmission by hyperpolarizing second order neurons due to increased K^+ conductance (for review, see Haas and Selbach, 2000).

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Adenosine A_1 receptors, however, are present in most areas of the brain (see Ribeiro et al., 2003 for a review) and supraspinal inhibitory activity of the adenosine A_1 system has been suggested by electroencephalography (Fulga and Stone 1998) and other techniques (Phillis et al., 1975; Uchimura and North 1991). Supraspinal antinociceptive actions of adenosine A_1 receptors are, however, poorly understood and although some studies showed that adenosine A_1 receptor agonists inhibit postsynaptic currents of periaqueductal grey neurons in brain slices (Bagley et al., 1999), the relative degree to which the effects of systemic adenosine A_1 receptor agonists are produced directly at supraspinal rather than at spinal sites remains unclear. It was therefore important to clarify this situation and we have made direct comparisons of the antinociceptive potency and efficacy of an adenosine A_1 receptor agonist in intact and spinalized animals with sensitization produced by carrageenan-induced inflammation. However, it has been shown that surgery related to the spinal column can enhance the direct spinal potency of some analgesic drugs on nociceptive reflexes (Herrero and Headley, 1991 and references within); we have, therefore, assessed the effect of the adenosine A_1 system in sham-spinalized animals. Also, some adenosine A_1 receptor agonists have been shown to be effective in the blockade of spinal cord segmental transmission in in vitro preparations, specially in immature rats (Nakamura et al., 1997). It is, therefore, possible that the inhibitory actions of adenosine A_1 receptor agonists depend not only on the type of preparation, but also on the level of development of the experimental animal, the degree of sensitization of the spinal cord, and the ligands used. It would be, therefore, interesting to compare the effectiveness of the same agonist and antagonist in an in vivo adult animal preparation and in an in vitro immature animal preparation in order to assess if the differences observed are due to the different ligands used or to the level of development of the animal used. We attempted to address this question, in the second part of this study, by studying the effect of CPA on the isolated hemisected spinal cord preparation from immature rats with and without carrageenan-induced inflammation.

2. Methods

2.1. In vivo experiments in adult rats

2.1.1. Preparatory surgery and groups of experiments

The antinociceptive activity of the adenosine A_1 receptor selective agonist CPA and the reversal effect of the selective antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT) were studied in electrophysiological experiments performed on adult male Wistar rats weighing 250–330 g, that were divided into three groups: intact (n=16), spinalized (n=6) and sham-spinalized animals (n=6). The preparatory surgery 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. In the groups of sham- and full-spinalized animals, a small laminectomy, with infiltration of lidocaine (1%) with adrenaline (10 μg/ml), was made from thoracic 10 to 8 vertebrae and the dura mater opened. In the group of sham-spinalized animals, no further surgery was performed and the incision was closed. In the group of spinalized animals, the spinal cord was sectioned at thoracic segment 8 or 9, depending on the level of vascularization, using cautery to minimize bleeding. After the surgery the animal was transferred to a frame, halothane was discontinued and anesthesia was maintained with α-chloralose (Sigma; 50 mg/kg for induction and 20 mg/kg/h by a perfusion pump for maintenance). The right hind limb was fixed into a Perspex block in inframaximal extension with plaster. The core temperature was maintained at 37±0.5 °C by means of a homeothermic blanket and blood pressure was monitored constantly. The preparation was left to rest for at least 1 h before the experiment started. In all the experiments, the systolic blood pressure was above 100 mm Hg before the administration of the drugs. All animals were injected in the right hind paw with 100 μl of carrageenan λ (Sigma) 10 mg/ml, in distilled water, intraplantar under brief halothane anesthesia (5% in oxygen for induction and 2% for maintenance), 16 h before the experiment. The effectiveness of carrageenan was studied by measuring the volume of the paw previous to the induction of inflammation and after the experiment by means of a plethysmometer (Letica).

2.1.2. Stimulus presentation and recording systems

The recording of withdrawal reflexes as single motor units has been described in detail several times and has been previously used to test the analgesic activity of different drugs (Herrero and Headley, 1991; Herrero et al., 1997; De Felipe et al., 1998; Romero-Sandoval et al., 2003; Gaitan et al., 2003). Briefly, units were activated by mechanical and electrical stimulation and recorded by means of a bipolar tungsten electrode inserted percutaneously into muscles of the right hind limb. Isolation of motor units was performed by moving the electrode with a micromanipulator while a mild pressure was applied to the paw. The activity was evoked in 3-min cycles consisting of 10 s of mechanical stimulation (0.2 N above the threshold over an area of 14 mm^2) and 16 electrical stimuli (20 ms width, 1 Hz, two times the threshold intensity for the recruitment of long latency responses; Herrero and Cervero, 1996a,b) applied to the most sensitive area of the cutaneous receptive field of the unit. Electrical stimulation was used to study the phenomenon of wind-up (see Herrero et al., 2000 for review). Mechanical stimulation was performed by a computer-controlled pincher device, and threshold force was considered as the minimum force needed to obtain sustained firing over the period of 10 s of stimulation. The

mean forces used for mechanical stimulation in the three experimental groups were 0.86±0.1 N in intact animals, 1.26±0.1 N in spinalized animals and 1.07±0.1 N in sham-operated animals. The mean intensity of electrical stimulation was 4±0.6 mA in intact animals, 5±0.7 mA in spinalized animals and 3.6±1 mA in sham-spinalized animals. At the end of the experiment, the animals were killed with an overdose of sodium pentobarbital (Euta-Lender, Normon).

2.1.3. Drugs and analysis of data

CPA (Sigma) was dissolved in dimethyl sulfoxide (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 two 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. 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 and the reversal of its effect was studied with CPT (Sigma) in 12 cases at doses of 10 and 20 mg/kg. Data are expressed as percentage of control, control being the mean of the three responses previous to the administration of the first dose. The quantitative analysis was based on counts of spikes evoked during the two cycles of stimulation between each dose. The data from the electrical stimulation were analyzed by counting the number of spikes evoked between 150 and 650 ms after each stimulus (C-fiber responses, Herrero and Cervero, 1996a,b). The protocol for stimulation and the collection of data were performed by computer, using commercial software (CED, UK; Spike 2). Statistical comparisons were made on raw data using commercial software (GraphPad-Prism and GraphPad-Instat for Windows). The one-way analysis of variance (ANOVA) with post-hoc Dunnett's test was used for the comparison between mechanical stimulation and control, for the analysis of wind-up curves, and for the analysis of blood pressure. Comparisons between the effect of CPA in different experimental groups were performed using the non-parametric Mann–Whitney *U*-test. The unpaired, two tailed *t*-test was used for the analysis of the level of inflammation. The data are presented as the mean±S.E.M.

2.2. In vitro experiments in immature rats

2.2.1. Animal preparation

Experiments were performed on isolated spinal cords with attached lumbar spinal dorsal and ventral roots using the grease-gap technique as described by Faber et al. (1997). Immature rats of either sex (2–6 days old, 8–20 g) were anesthetized with urethane (1.5 g/kg, i.p.), and following decapitation the spinal cord was removed, hemisected along the midline and placed into a recording chamber. L4 or L5 dorsal root and the corresponding ipsilateral ventral root were in contact with a stimulating and a recording electrode, respectively. Electrical isolation was achieved with grease gaps using a mixture of vaseline and paraffin oil. The preparation was superfused at a rate of 2 ml/ min with artificial cerebrospinal fluid (ACSF) composed of (in mM) NaCl (118), KCl (3), $CaCl_2$ (1.5), $mgSO_4$ (1.25), $NaHCO_3$ (24) and glucose (12). The ACSF was continuously gassed with O_2/CO_2 (95%/5%) and maintained at room temperature at a pH of 7.4. The preparation was allowed to equilibrate for at least 60 min before any testing commenced.

2.2.2. Electrophysiological recordings

Electrical square pulses (0.5 ms width) were applied to the dorsal root to determine the threshold (T) at which the first discernible wave was recorded from the corresponding ipsilateral ventral root. Three different protocols of electrical stimulation were used: low intensity stimuli (single pulses at 3 T intensity), high intensity stimuli (single pulses at 16 T intensity) and repetitive high intensity stimuli (16 pulses at 16 T intensity, 1 Hz). Separate control experiments were performed in isolated spinal nerve-dorsal root preparations as described in a previous study (Faber et al., 1997) to ensure that the low intensity stimulation activated only A-fibers, and the high intensity both A- and C-fibers in the peripheral nerve (data not shown). Different components of dorsal root-evoked ventral root potentials were observed. Low intensity stimulation evoked an initial monosynaptic action potential (monosynaptic reflex, Fig. 5), with a maximal duration of 200 ms (Faber et al., 1997), that was superimposed on a polysynaptic excitatory postsynaptic potential (slow ventral root potential) which lasted for up to 4 s (see an example in Fig. 5). Single high intensity stimuli evoked a prolonged A- and C-fiber-mediated polysynaptic excitatory postsynaptic potential (slow ventral root potential, Fig. 6) with a duration of about 40 s. Trains of high intensity stimuli evoked a C-fiber-mediated wind-up response with a duration up to 50 s (Fig. 6). Averages of five consecutive low intensity sweeps (interpulse interval 6 s) followed by averages of five consecutive single (interpulse interval 18 s) and repetitive high intensity sweeps (interpulse interval 22 s) were obtained. This stimulation sequence was applied 20 min after the onset of drug perfusion at the respective concentration. Signals were digitized, stored and analyzed using commercially available software (DASYLAB, Synotech, Germany). The parameters analyzed in the evoked responses were the peak amplitude of the monosynaptic reflex and the area under the curves of the low intensity slow ventral root potential, high intensity slow ventral root potential and wind-up. Drug testing commenced only if responses in three control cycles showed a variability lower than 20%. Drugs were dissolved in cremophor, diluted in ACSF and applied via the perfusate. Several concentrations were tested on the same preparation in a cumulative manner, with equilibration periods sufficient to reach a plateau of the effect (typically 20 min).

2.2.3. Behavioral experiments

Mechanical hyperalgesia was induced 16 h before the experiment by an intrajoint injection of carrageenan (30 mg/ml, 20 μl, under halothane anesthesia) into the ankle of the left hind paw. Control experiments were made by injecting a similar amount of saline. Withdrawal thresholds to mechanical stimulation were measured before the injection of carrageenan and prior to the experiment using an electronic von Frey filament (tip diameter of 0.76 mm, Model 1601, IITC/Life Science Instruments). Five tests, with a cut off force of 45 g, were made on each paw with at least a 30-s of interval between them.

2.2.4. Drugs and data analysis

Drug effects were calculated as mean percentages of control value, control being the average of the last three pre-drug responses. The one-way ANOVA with post-hoc Dunnett's test was used for statistical comparisons. Data are expressed as mean±S.E.M.

All the experimental procedures conformed to the institutional, national and European guidelines for the use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3. Results

3.1. In vivo experiments in adult rats

3.1.1. Effect of CPA on responses to noxious mechanical stimulation

Fig. 1 shows pooled data of the effects observed after the intravenous administration of CPA on single motor unit responses to noxious mechanical stimulation in spinalized, intact and sham-spinalized animals. The administration of CPA in spinalized animals did not modify the single motor unit responses to noxious mechanical stimulation. In fact, doses from 10 to 160 μg/kg induced a non-significant increase of responses up to a maximum of 121±12% of control response with the dose of 160 μg/kg.

CPA dose-dependently reduced responses to noxious mechanical stimulation in intact animals with a minimum effective dose (MED) of 160 μg/kg (P<0.01) and an ID50 of 92±1.3 μg/kg. The maximum effect observed was of 23±12% of control response. The effect of CPA was monitored for a 45-min period in four experiments and no recovery of the inhibition of responses was observed during this time (9.5±8%, P<0.01). The administration of equivalent doses of vehicle, using the same protocol as for CPA (n=3), did not cause any significant change in responses evoked by noxious mechanical stimulation (107±13% of control response with the highest dose).

In sham-spinalized animals, the administration of CPA induced a dose-dependent inhibition of responses that was more intense than that observed in intact animals (Fig. 1). In this case, the effect was significant from the dose of 10 μg/kg (P<0.01) with an effect of 46±8% of control. The ID50 could only be calculated by approximation, giving a value of 8.3±1 μg/kg, more than 10-fold lower than that observed in intact animals (P<0.001). The reduction of responses observed was significantly lower than that seen in the group of intact animals from doses of 10 to 160 μg/kg (Fig. 1). The maximum observed effect was 6±3% at the highest dose tested.

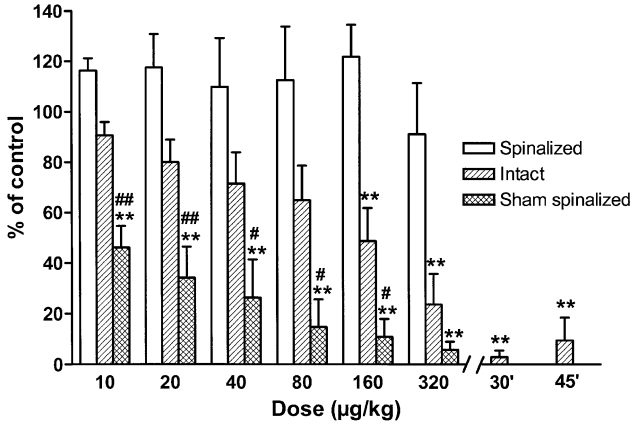

Fig. 1. Pooled data of single motor unit responses evoked by noxious mechanical stimulation after the administration of cumulative doses of CPA in spinalized, intact and sham-spinalized animals. The administration of CPA in spinalized animals induced a non-significant increase of the responses, whereas CPA dose-dependently reduced responses in intact animals. The maximum effect was, however, observed in sham-spinalized animals. (**P<0.01, comparison vs. control response using the one-way ANOVA, with the post-hoc Dunnett test; #P<0.05; ##P<0.01, intact vs. sham-spinalized using the non-parametric Mann–Whitney U-test).

3.1.2. Effect of CPA on responses to electrical stimulation (wind-up)

As in previous studies (Herrero and Cervero, 1996a,b), the maximum firing rate of control responses observed in spinalized animals (8±1) was lower than that seen in intact animals (11±1). In sham-operated animals, this firing rate was in between these two values (9±2); these differences, however, were not statistically significant. The administration of CPA was effective in reducing wind-up responses in intact and sham-spinalized animals but not in full-spinalized animals (Fig. 2). As for responses to noxious mechanical stimulation, the administration of CPA to spinalized animals caused a non-significant and non-dose-dependent enhancement of wind-up responses with all the doses studied (Fig. 2). In intact animals, CPA induced a significant and dose-dependent reduction of wind-up. The MED observed was 160 μg/kg (P<0.05) and the maximum effect was observed with the highest dose tested: 58±13% of control response (P<0.01). In sham-spinalized animals (Fig. 2), the administration of CPA induced a potent reduction of the wind-up curve, observing a significant effect from the first dose used of 10 μg/kg (P<0.01). The maximum effect was observed at the dose

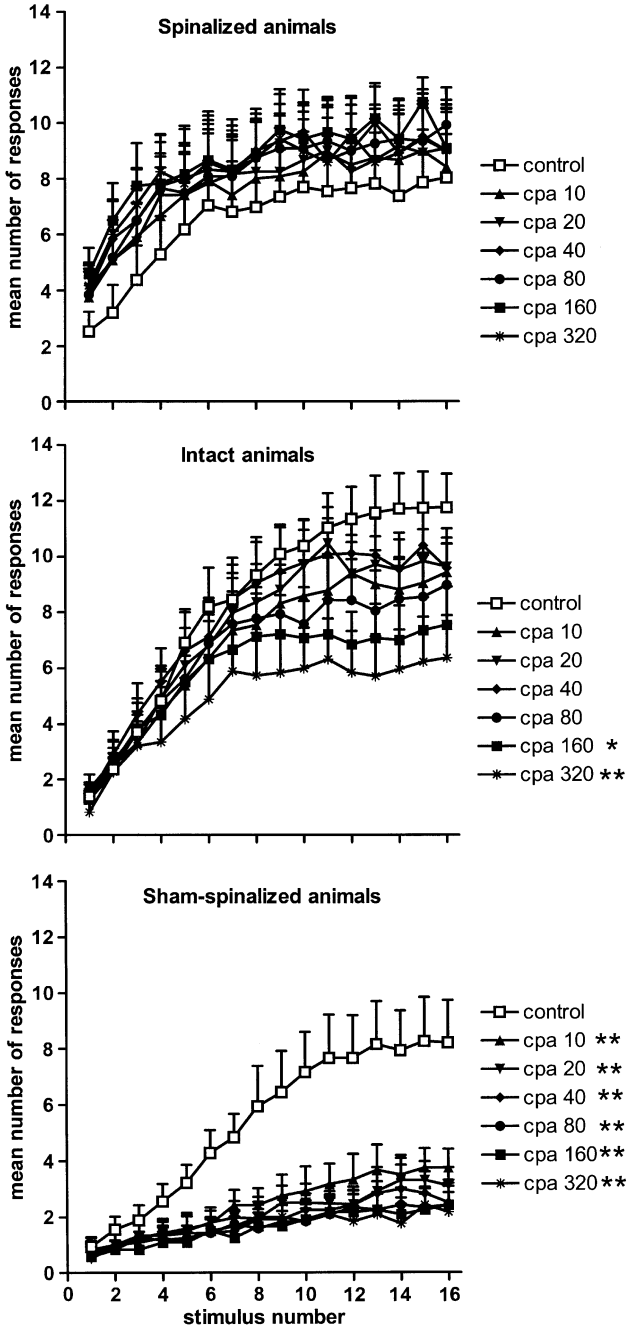

Fig. 2. Single motor unit wind-up before and after the administration of cumulative doses of CPA. No reduction of wind-up was observed in spinalized animals. In intact animals CPA induced a significant and dose-dependent reduction of wind-up. The effect observed in sham-spinalized was more intense than in intact animals. Statistical comparison between responses observed with each dose and the control response was made using the one-way analysis of variance, ANOVA, with the post-hoc Dunnett test, *P<0.05; **P<0.01).

of 160 μg/kg (36±11% of control, P<0.01) and was not significantly different from that observed with 320 μg/kg (41±15%, P<0.01). The administration of equivalent doses of vehicle induced a non-dose-dependent enhancement of the wind-up activity, with a maximum of 152±83% of control response.

3.1.3. Reversal of the antinociceptive effect of CPA by CPT

The antinociceptive activity observed by the administration of CPA in intact and sham-spinalized animals was challenged with the intravenous administration of the adenosine A_1 receptor antagonist CPT at two doses each of 10 mg/kg (total cumulative dose of 20 mg/kg). CPT was injected 6 and 12 min after CPA and its effect was studied for 18 min, during which the effect of CPA was still complete in the absence of antagonist (Fig. 1). In intact animals (n=12), the reversal effect of CPA was variable and two groups of units were observed. In one group of units (n=6), the inhibitory effect of CPA was not reversed by the administration of CPT, in responses to noxious mechanical stimulation (20±7% of control, P<0.01 vs. control, Fig. 3A) and only partially reversed in response to electrical stimulation (74±4% of control, P<0.05 vs. control response). In a second group of units (n=6), full recovery of CPA effect was observed 18 min after the administration of 20 mg/kg of CPT, either in response to noxious mechanical stimulation (90±3% of control, Fig. 3A) or to wind-up (102±6% of control). In sham-spinalized animals, the administration of CPT at a similar dosage only partially reversed the inhibition of responses to noxious mechanical stimulation and wind-up (32±11% and 50±12% of control, respectively).

3.1.4. Effect of CPA on blood pressure

Even though the administration of CPA was made very slowly, a significant drop in blood pressure was always observed after the injection of each dose. This drop was dose-dependent and very similar in the three experimental groups (Fig. 3B) with maximal decreases of 43±5% in spinalized animals, 37±4% in intact animals and 42±4% in sham-operated animals (P<0.01 vs. control response, in all cases). Mean arterial pressure was monitored for up to 45 min in three intact animals and only a slight and not significant recovery was observed at this time (54±10%).

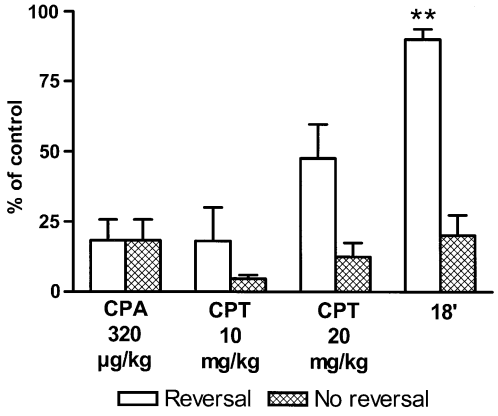

Fig. 3. Reversal of the effect of CPA by CPT on responses evoked by noxious mechanical stimulation in intact animals. The administration of CPT induced a full reversal of the CPA effect in 50% of the units (90±3% of control), whereas no effect was observed in another 50% of the units (20±7% of control).

The administration of CPT at a dose of 10 mg/kg, 6 min after the last dose of CPA, reversed the decrease in blood pressure almost completely: 73±5% in spinalized animals, 71±6% in intact animals and 83±2% in sham-spinalized animals. A second dose of 10 mg/kg of CPT (cumulative dose of 20 mg/kg) did not further increase the blood pressure level significantly (Figs. 3B and 4).

3.2. In vitro experiments in immature rats

3.2.1. Effect of CPA on the monosynaptic reflex and the low intensity slow ventral root potential

Animals treated with carrageenan displayed an intense mechanical hyperalgesia 16 h after the induction of inflammation. The withdrawal threshold for mechanical stimulation prior to the induction of inflammation was 18±2 g, whereas it was only 9±3 g (n=6, P<0.05) 16 h after the administration of carrageenan. No changes were observed in the threshold of the contralateral paw, nor in animals treated with saline (19.4±3.9 vs. 19.5±5.2 g, n=6).

CPA induced a dose-dependent inhibition of the monosynaptic reflex and the low intensity slow ventral root potential (Fig. 5), in both animals with and without inflammation (n=6, each). The IC50s in animals treated with carrageenan were 9±0.7 nM (monosynaptic reflex) and 7.7±1.3 nM (slow ventral root potential), with maximum effects of 7±5% (P<0.01) and 20±4% of the control response (P<0.01, Fig. 5), respectively. In animals treated with vehicle, the IC50s were of 12.3±1.2 nM in monosynaptic reflex and 7.9±1.5 nM in slow ventral root potential, with a maximum effect of 21±10% of control in monosynaptic reflex (P<0.01) and of 23±8% in slow

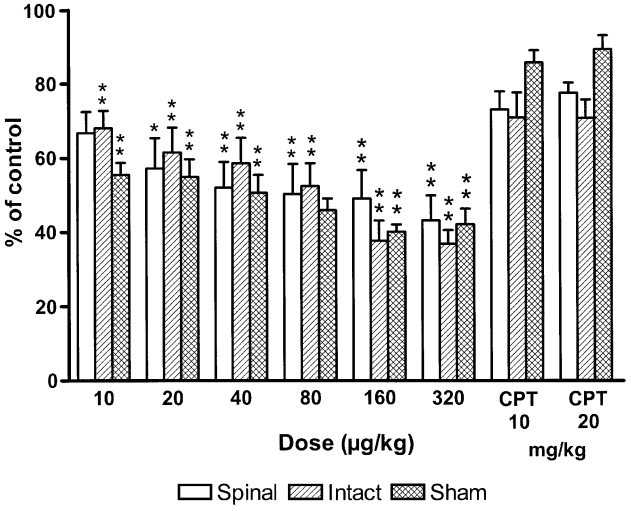

Fig. 4. Effect of CPA on mean arterial pressure. An important drop in blood pressure was always observed after the injection of each dose of CPA. The effect observed was very similar en the three experimental groups with all the doses tested. In spinalized animals, the maximal decrease was of 43±5% of control (P<0.01). In intact animals, the maximal reduction was of 37±4% (P<0.01) and in sham-spinalized of 42±4% (P<0.01). The administration of CPT reversed the effect in all cases (statistical comparison and layout as for Fig. 1).

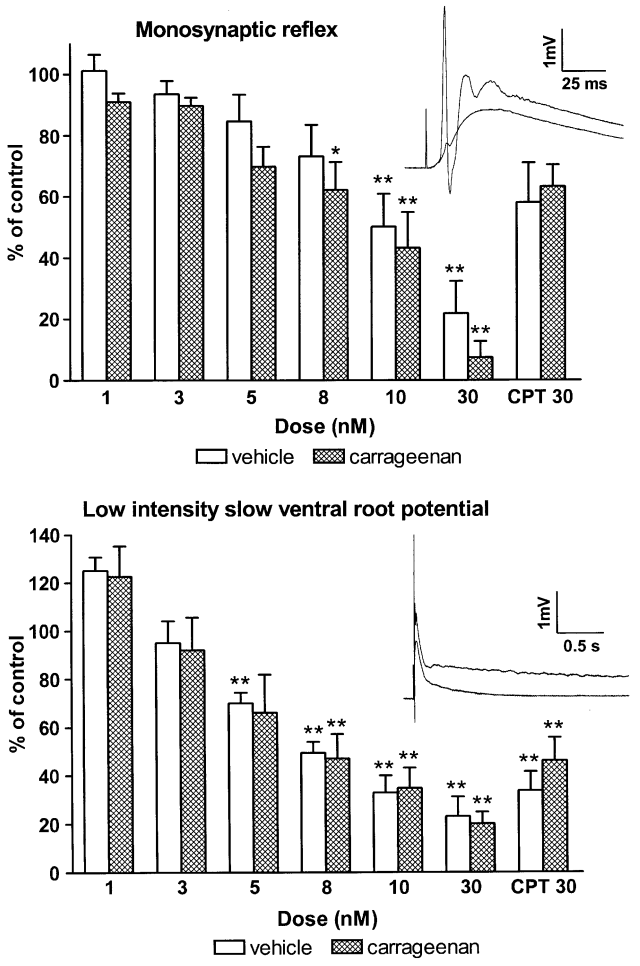

Fig. 5. Pooled data of the effects of CPA on responses to low intensity electrical stimulation: monosynaptic reflex and low intensity slow ventral root potential, in the hemisected and immature spinal cord preparation. CPA induced a dose-dependent inhibition of the monosynaptic reflex and the low intensity slow ventral root potential either in animals with or without inflammation. The administration of 30 nM of CPT induced a partial recovery of the inhibition of monosynaptic reflex but not of the effect observed in the low intensity slow ventral root potential. Insets show representative traces of control curves and 30 nM CPA effect (statistical comparison and layout as for Fig. 1).

ventral root potential (P<0.01). No significant differences were observed between the two experimental groups. The administration of 30 nM of the antagonist CPT induced a partial reversal of the inhibition of monosynaptic reflex, but not of the effect observed on the low intensity slow ventral root potential (Fig. 5).

3.2.2. Effect of CPA on the high intensity slow ventral root potential and in wind-up

Similar to the responses to low intensity electrical stimulation, the administration of CPA induced a dose-dependent inhibition of the slow ventral root potential evoked by high intensity stimulation and of wind-up (Fig. 6). The maximum effects observed in animals with inflammation were 7±1% for slow ventral root potential and 28±7% for wind-up, with IC50s of 4.9±0.5 and 12±2

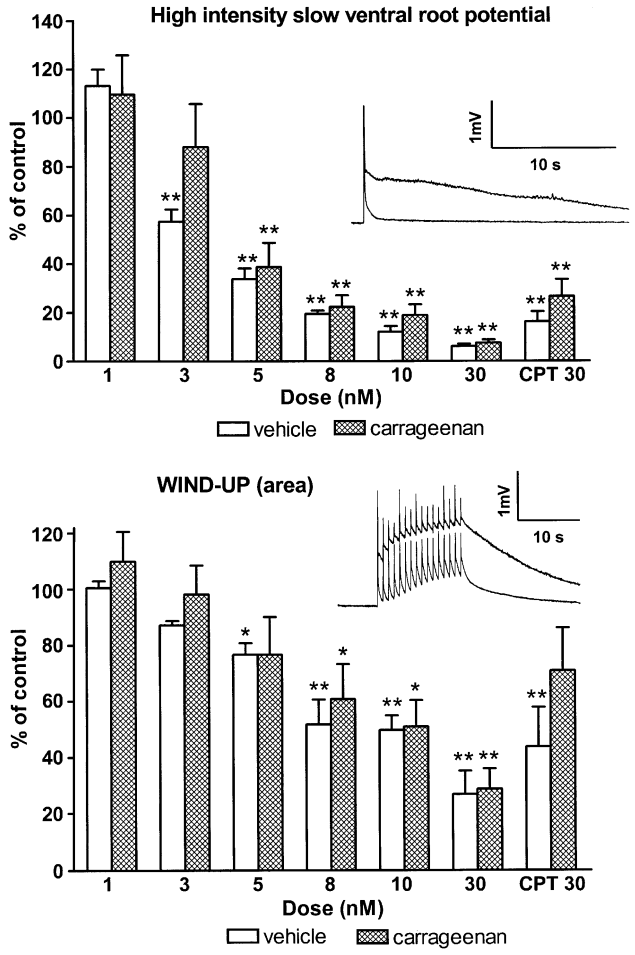

Fig. 6. Pooled data of the effects of CPA on responses to high intensity electrical stimulation: slow ventral root potential and wind-up, in the hemisected and immature spinal cord preparation. As in responses to low intensity stimulation, CPA was very effective in reducing segmental transmission in the spinal cord with no significant differences between animals with or without inflammation. CPT was not effective in the reversion of the effect at the doses tested. Insets show representative traces of the different components of the control response to high intensity stimulation and the effect of 30 nM CPA (statistical comparison and layout as for Fig. 1).

nM, respectively. In animals without inflammation, the maximum reduction of the responses was 6±1% for slow ventral root potential and 27±8% for wind-up, with IC50s values similar to those observed in animals with inflammation: 3.8±0.5 nM for slow ventral root potential and 10.7±1.2 nM for wind-up. The administration of CPT was not effective in reversing the inhibition (Fig. 6).

4. Discussion

The main observation made in the present experiments is that the systemic administration of CPA in adult intact animals induced an intense and dose-dependent inhibition of nociceptive responses to either mechanical or high intensity repetitive electrical stimulation, and this effect lasted for at least 45 min. In spinally transected animals, however, the administration of CPA did not modify the single motor unit nociceptive responses, suggesting that CPA is very effective as antinociceptive agent in the whole adult animal but, also, that the integrity of the spinal cord is crucial to observe this effect when injected systemically. Thus, the antinociceptive activity of CPA in the present experimental conditions is, at least in part, located supraspinally and is supported by previous studies showing supraspinal effects of the adenosine systems. The administration of adenosine A_1 receptor agonists into the medial pontine reticular formation, for example, induces a potent antinociceptive effect (Tanase et al., 2002). Adenosine is actively released in the supraoptic nucleus (Oliet and Poulain, 1999), inhibits excitatory transmission in the laterodorsal tegmentum (Arrigoni et al., 2001) and is a very effective analgesic drug after i.c.v. administration (Zarate et al., 1999).

These observations do not necessarily exclude a direct action of CPA in the spinal cord. In fact, adenosine A_1 receptor-mediated antinociception has also been reported in behavioral tests after intrathecal administration (see Sawynok, 1998; Ackley et al., 2003; Sawynok and Liu, 2003 for review), and the dorsal horn and primary afferents contain adenosine A_1 receptors (see Sawynok, 1998; Ackley et al., 2003; Sawynok and Liu, 2003 for review). Similar supraspinal activity has been shown with the systemic administration of opiates (Yeung and Rudy 1980a,b), although spinal cord activity of opiates was also evident, and a synergistic interaction between a supraspinal and a spinal activities occurred when opiates were given discretely to the two compartments in awake animals (Yeung and Rudy 1980b). A similar supraspinal/spinal "self-synergy" was observed with acetaminophen (Raffa et al., 2000) and the results observed in our experiments might also be the result of an interaction of CPA-mediated antinociceptive activity at spinal and supraspinal sites. It is also possible that the anesthetic used in the present experiments contribute to this possible synergistic action or to an enhancement of CPA activity, however, although further experiments are needed to elucidate this question, CPA was not effective at all in spinalized animals, suggesting a lack of interaction between chloralose and CPA in the present experimental conditions.

In addition, we observed a significant reduction of wind-up by CPA, an effect that has been described in dorsal horn neurons after the intrathecal administration of CPA (Reeve and Dickenson, 1995). This effect on wind-up supports an action on the spinal cord (see Herrero et al., 2000 for review). However, wind-up is also strongly modulated by supraspinal influences (Herrero and Cervero, 1996a,b), as is the response of spinal wide dynamic range neurons to noxious stimulation (Dugast et al., 2003; Almeida et al., 2000). The depression observed might be the result of the activation of a descending inhibitory control, supported by the strong enhancement of the antinociceptive activity of CPA observed in sham-spinalized animals. An increase of the effect of some drugs after

sham spinalization has been observed in similar experiments performed with μ and κ opioid receptor agonists (Herrero and Headley, 1991, 1993) but not with other compounds (Romero-Sandoval et al., 2003). An explanation for this observation is that the surgery sensitizes some supraspinal sites to the actions of CPA or, in turn, that this surgery activates some supraspinal systems that enhance the potency of CPA. In any case, the transection of the spinal cord eliminates this action and all observed antinociceptive effects of CPA, indicating that the primary effect of systemically administered CPA is supraspinal.

Experiments performed in in vitro preparations on isolated spinal cords show an effect of some adenosine A_1 receptor agonists on the spinal cord segmental transmission (Nakamura et al., 1997), indicating that, under these conditions, supraspinal influences are not needed for some of the inhibitory actions of the adenosine A_1 receptor system. A proper comparison of our data with these studies was not possible since the type of inflammation and the agonists used were different. For this reason, we conducted experiments in isolated in vitro preparations from newborn rats to compare the effect of the same drugs, CPA and CPT, in a similar state of inflammation. These experiments show that CPA inhibits segmental low and high intensity transmission in the hemisected spinal cord preparation in a dose-dependent manner. These data are in agreement with the inhibitory effects observed with two other adenosine A_1 receptor agonists (cyclohexyladenosine, CHA and R-phenylisopropyladenosine, R-PIA) in previous experiments (Nakamura et al., 1997).

Our experiments, therefore, suggest that CPA is an effective inhibitory drug in the immature isolated spinal cord preparation, which is not yet influenced by a completely developed supraspinal control. In contrast, in adult animals, in which descending modulation of spinal cord somatosensory processing is well developed, supraspinal influences determine the antinociceptive effect of CPA.

In the immature spinal cord preparation, we observed no change in the dose–response curve of CPA-mediated inhibition of spinal transmission in the spinal cord from rats with a carrageenan-induced inflammation as compared to control rats. This is in agreement with data from similar experiments, and in contrast to previous observations in adult animals (Poon and Sawynok, 1998), where an adenosine A_1 receptor agonist was more potent in inhibiting responses on the inflamed side compared to the non-inflamed side using the carrageenan thermal hyperalgesia model. The expression of adenosine A_1 receptor mRNA and protein has been shown to be regulated in neuronal and non-neuronal preparations by different factors, such as glucocorticoids, cisplatin, thyrotropin and by the adenosine A_1 receptor agonist CPA itself (Svenningsson and Fredholm, 1997; Hettinger et al., 1998; Bhat et al., 1999; Vainio et al., 2000). Apart from the fact that spinal adenosine A_1 receptors have been shown to be down-regulated by chronic intracerebroventricular administration of morphine (Tao et al., 1995), it has not been reported whether their expression changes under pathological conditions. However, Suzuki et al., (2000) described an enhanced effectiveness of intrathecal CPA after spinal nerve injury, thus supporting an altered functional expression of spinal adenosine A_1 receptors associated with neuropathy. However, in agreement with our results a lack of regulation of adenosine A_1 receptor mRNA in lumbar segments L4–L6 has been observed 2 days after the induction of plantar inflammation by the administration of complete Freund's adjuvant (Clemens Gillen, Grünenthal, unpublished observation). A possible explanation for this mismatch is that changes in the adenosine A_1 receptor, and its sensitivity for its ligands, occur in situations of neuropathy but not in carrageenan-induced inflammation. Nevertheless, further experiments are needed to elucidate this question.

The reversal of the antinociceptive activity of CPA was not observed in all cases after the administration of CPT in adult animals and only partially in immature rats. CPT is a selective antagonist of adenosine A_1 receptors (Bruns, 1981) and it has been shown to be an active and selective blocker of the adenosine A_1 receptor-mediated antinociceptive actions when administered intrathecally (Lee and Yaksh, 1996). In an in vitro preparation, Oliet and Poulain (1999) showed that adenosine can be released within the supraoptic nucleus at a concentration sufficient to inhibit the release of γ-aminobutyric acid (GABA) and glutamate, and this effect was reversed by the application of low doses of CPT. Also, the inhibition of evoked excitatory transmission in the laterodorsal tegmentum by adenosine is antagonized by CPT (Arrigoni et al., 2001). All this suggest that the partial or lack of effect of CPT in our experiments should not be due to a lack of selectivity of the antagonist. It is possible that CPT did not get full access to central sites, however, although a different ability to cross the blood brain barrier depending on the stage of development of the brain is possible, this does not seem likely since partial reversal was also observed in the in vitro preparation. It is also possible that the effect of CPA was not only mediated by the adenosine A_1 receptor but also by other systems. The reduction of spinal excitability by the activation of adenosine A_1 receptors has been suggested to be secondary to a modulation of the glutamatergic transmission (Patel et al., 2001; Ackley et al., 2003). An interaction with glutamatergic transmission is supported by the intense reduction of the wind-up observed in all the experiments performed in our study, a phenomenon mediated by the *N*-methyl-D-aspartic acid (NMDA) receptor (Herrero et al., 2000 for review). There are also data supporting a common signaling pathway for μ-opioid, α2 and adenosine A_1 receptors in the periphery, involving protein kinase C as the second messenger system (see Aley and Levine, 1997; Sawynok and Liu, 2003 for review). In central areas, adenosine A_1 receptors seem to mediate the antinociception induced by the supraspinal administration of β-endorphin but not morphine in rodents (Suh et al., 1997). Also, the

spinal antinociceptive activity of morphine was enhanced in an additive manner by spinal adenosine in a rat model of neuropathic pain, and the spinal injection of adenosine deaminase or reuptake inhibitors greatly enhanced the effect of spinal morphine (Lavand'homme and Eisenach, 1999). In summary, under the present experimental conditions, the antinociceptive activity of CPA was possibly due, not only to a primary action at adenosine A_1 receptors, but also to the secondary activation of another system or even to a variable access to the site of action.

Cardiovascular effects of adenosine are well known and described in detail in many studies. The action of adenosine on cardiovascular control seems to be exerted in areas of the brainstem (Thomas and Spyer, 1999), though an action on sympathetic spinal cord neurons has also been shown (Deuchars et al., 2001). In the present experiments, the administration of CPA induced an intense hypotension in all three experimental groups. The effect was dose-dependent and the intensity was very similar in all cases, indicating the lack of influence of supraspinal systems in the effects of CPA on the cardiovascular system. The administration of low doses of CPT reversed the hypotensive effect mediated by CPA, suggesting that selective action on adenosine A_1 receptors is responsible for this effect. It is important to point out that the cardiovascular effect did not parallel the antinociceptive effect observed by the administration of CPA. Whilst the hypotension observed was similar in the three groups of experiments, the antinociceptive actions were very different, ranging from no effect in spinalized animals to a very potent inhibition of the nociceptive responses in sham-spinalized animals. This rules out the possibility that the antinociceptive effects of CPA were a result of the depression of the nervous system as a result of the hypotension. This is also supported by the observation that CPT was very effective in reversing the cardiovascular effects produced by CPA, but it was not that effective on the inhibition of nociceptive responses. On the other hand, the inhibition of nociceptive responses was still observed after the recovery of the blood pressure. It seems, therefore, that the cardiovascular and antinociceptive effects of CPA are due to a different mechanism of action and, while the integrity of the spinal cord is crucial in order to observe CPA-mediated antinociceptive effects, the cardiovascular actions are not influenced by the spinalization or spinal surgery and are, therefore, related to a supraspinal-independent effect.

In conclusion, this study appears to be the first to show that the antinociceptive effect of systemic CPA depends on the integrity of the spinal cord in the adult animal, but not in the immature rat, and varies with spinal cord-related surgery. This is supported by the intense enhancement of the antinociceptive activity induced by the spinal column surgical trauma. The variability observed on the effect of CPT in spinal cord transmission suggests an involvement of other adenosine A_1-independent systems in the antinociception observed.

5. Uncited reference

Hartell and Headley, 1991

Acknowledgments

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