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Antinociceptive and anti-inflammatory properties of an adenosine kinase inhibitor and an adenosine deaminase inhibitor

Anthony Poon [*,1], Jana Sawynok [1]

Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

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Abstract

Spinal administration of an adenosine kinase inhibitor, alone or in combination with an adenosine deaminase inhibitor, produces antinociception in inflammatory pain tests. In the present study, we examined the antinociceptive and anti-inflammatory effects produced by the peripheral (intraplantar) administration of 5′-amino-5′-deoxyadenosine (an adenosine kinase inhibitor), 2′-deoxycoformycin (an adenosine deaminase inhibitor), and combinations of both agents in the carrageenan-induced thermal hyperalgesia and paw oedema model in the rat. When injected in the *ipsilateral* paw immediately prior to carrageenan injection, both agents produced antinociception only at the highest dose (1 μmol), whereas a reduction in paw swelling was evident at a lower dose (300 nmol). Significant augmentation in both the antinociceptive and anti-inflammatory effects was seen when 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin were co-administered in equimolar doses at all dose levels. Both effects were mediated via activation of adenosine receptors, as indicated by blockade by an adenosine receptor antagonist. When administered into the *contralateral* paw, 1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin produced prominent antinociception, indicating a systemic drug activity. There was only a modest reduction in paw oedema in the carrageenan-injected (ipsilateral) paw, suggesting that much of this activity was locally mediated. Reversal of systemic effects on thermal thresholds by an intrathecal adenosine receptor antagonist implicates a spinal site of action in this instance. An *ipsilateral* administration of 1 μmol 5′-amino-5′-deoxyadenosine, but not 1 μmol 2′-deoxycoformycin, reduced carrageenan-induced c-Fos expression in the spinal dorsal horn, and this was further reduced by the peripheral co-injection of the two agents. These results provide evidence for a predominantly *spinal* antinociceptive effect and a predominantly *peripheral* anti-inflammatory effect produced by inhibitors of adenosine kinase and adenosine deaminase.

Keywords: Antinociception; Anti-inflammatory; Adenosine kinase; Adenosine deaminase

1. Introduction

Adenosine produces complex effects on peripheral pain signaling by actions on the sensory nerve terminal, and its involvement in the inflammatory process potentially produces secondary effects on pain signaling. In behavioural studies, administration of selective adenosine A_1 receptor agonists directly into the rat hindpaw produces antinociception in a mechanical hyperalgesia (paw pressure) model (Taiwo and Levine, 1990), as well as in the formalin test (Karlsten et al., 1992; Doak and Sawynok, 1995). In contrast, activation of the adenosine A_2 receptor appears to be pronociceptive as peripheral administration of selective adenosine A_2 receptor agonists produces mechanical hyperalgesia (Taiwo and Levine, 1990) and enhances nociception produced by formalin (Karlsten et al., 1992; Doak and Sawynok, 1995); pronociceptive effects produced by 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS21680) specifically suggest the involvement of the A_2 receptor subtype (Doak and Sawynok, 1995). Like the adenosine A_1 receptor, the adenosine A_2 receptor appears to be located directly on the sensory afferent nerve terminal (Taiwo and Levine, 1990). More recently, the adenosine A_3 receptor has been suggested to play a pronociceptive role, possibly indirectly via the release of histamine and 5-hydroxytryptamine from mast cells (Sawynok et al., 1997), which are found adjacent to sensory nerve terminals (Coderre et al., 1989).

* Corresponding author. Department of Endodontics, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284, USA. Tel.: +1-210-567-6668; fax: +1-210-567-4209.

E-mail address: poona@uthscsa.edu (A. Poon)

[1] Tel.: +1-902-494-2596; fax: +1-902-494-1388.

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Although the role of adenosine in inflammation is complex, there is increasing evidence to support the hypothesis that adenosine serves as an endogenous anti-inflammatory agent (reviewed Cronstein, 1994, 1997). Release of adenosine by neutrophils and endothelial cells has been shown both in vitro (Cronstein et al., 1983) and in vivo (Rosengren et al., 1991). Suppression of inflammation by adenosine is mainly due to inhibition of adenosine A_{2A} receptors on inflammatory cells, especially neutrophils. Thus, adenosine agonists attenuate neutrophil-mediated endothelial cell injury (Cronstein et al., 1986), inhibit the generation of the superoxide anion by stimulated neutrophils (Cronstein et al., 1983), as well as inhibit endotoxin-stimulated production of tumor necrosis factor-α, a cytokine which promotes neutrophil accumulation and function (Thiel and Chouker, 1995). These effects were found to be mediated by activation of the A_{2A} adenosine receptor. Anti-inflammatory properties of endogenous adenosine are also observed in in vivo studies. Thus, Rosengren et al. (1991) demonstrated an enhancement in inflammation in the hamster cheek pouch model by 8-phenyltheophylline, an adenosine receptor antagonist. The anti-inflammatory actions of methotrexate and sulfasalazine have also been attributed, at least in part, to an increase in endogenous adenosine levels (Cronstein et al., 1993; Gadangi et al., 1996).

Inhibitors of adenosine kinase and adenosine deaminase have been developed both as investigative tools to study the properties of the enzymes and as potential therapeutic agents. Recruitment of endogenous adenosine to produce therapeutic effects could be especially useful when the pathological process involves site- or event-specific adenosine release, either as a side-effect or as an attempt by the system to limit the extent of injury. Behavioural effects of these agents have been examined following spinal administration, but there are few data following local administration. Thus, the intrathecal administration of 5′-amino-5′-deoxyadenosine, but not of 2′-deoxycoformycin, induces antinociception in a thermal threshold test (Keil and DeLander, 1992), the formalin test (Poon and Sawynok, 1995), and the carrageenan thermal hyperalgesia test (Poon and Sawynok, 1998); reversal of this action by methylxanthines indicates the involvement of spinal adenosine receptors. When co-administered intrathecally, these agents produce augmented antinociceptive effects (Poon and Sawynok, 1998), and enhance spinal adenosine-induced antinociception (Keil and DeLander, 1994). In neurochemical studies, 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin both enhance release of adenosine in vitro and in vivo, and combinations produce a greatly enhanced release of adenosine (Golembiowska et al., 1995, 1996). Administered to the hindpaw, some local antinociceptive actions for 5′-amino-5′-deoxyadenosine, 2′-deoxycoformycin and combinations thereof have been observed in the formalin test (Sawynok et al., 1998).

The current study examined the potential peripheral antinociceptive and anti-inflammatory properties of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin, both when injected alone and in combination into the rat hindpaw, in a carrageenan-induced thermal hyperalgesia and paw oedema model. Drugs were administered to either the ipsilateral (carrageenan injected) or contralateral hindpaw in order to determine whether drug effects were peripherally or systemically mediated. The possibility of a spinal site of action for systemic effects was examined by the intrathecal administration of caffeine, a non-selective adenosine antagonist. Fos-like immunoreactivity in the spinal cord has been demonstrated after induction of paw inflammation by carrageenan (Noguchi et al., 1991), and c-Fos expression has been widely used as a marker in studying the effects of classical and putative analgesic compounds (e.g., Abbadie and Besson, 1993; Buritova et al., 1996a,b; Honoré et al., 1996). Thus, the effects of peripherally administered 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin on carrageenan-induced spinal c-Fos expression also were investigated in the present study.

2. Materials and methods

2.1. Animals

All experiments were carried out using male Sprague–Dawley rats (Charles River Laboratories, Quebec, Canada). Rats weighing 100–125 g and 300–325 g were used in behavioural experiments, the latter being used when an antagonist was administered via a surgically implanted intrathecal catheter. For the measurement of carrageenan-induced spinal c-Fos expression, 225–300 g rats were used. Rats were housed in groups of two to three, except for those with implanted intrathecal catheters which were housed individually following catheterization. All rats were kept in an animal care facility maintained at 21°C–23°C on a 12 h light/dark cycle and were given free access to food and water. Experimental procedures were approved by the University Committee for Laboratory Animals and deemed to be in accordance with the Canadian Council on Animal Care guidelines, as well as guidelines on the use of animals in pain research provided by the International Association for the Study of Pain (IASP). Each animal was tested only once.

2.2. Thermal hyperalgesia testing

Thermal hyperalgesia testing was performed following the method described by Hargreaves et al. (1988). Rats were placed individually in clear plastic chambers (9 cm × 22 cm × 25 cm) on a glass surface, the temperature of which was maintained at 30°C using a thermal probe and an electronic feedback circuit. Animals were allowed to acclimatize for 30 min before baseline measurements were performed. The heat source consisted of a high-intensity projector lamp bulb (Sylvania 50 W, 8 V) placed under-

neath the glass floor and projecting through a 5-mm diameter aperture. To ensure consistency as well as to avoid the less sensitive tori, the aperture was always positioned under the mid-hindpaw before activation of the heat source. The bulb and an electronic timer were simultaneously activated upon initiating a test, and both were automatically inactivated when photoelectric cells mounted adjacent to the aperture detected a change in the reflected light as a result of paw withdrawal in response to the heat. The paw withdrawal latency to the nearest 0.1 s was recorded. To prevent tissue damage, the cut-off latency was set at 20.5 s, a value approximately 2.5 times that of baseline values in normal paws (8 s).

2.3. Measurement of paw oedema

Paw volume in milliliters was measured using a plethysmometer (Ugo Basile). The hindpaw was immersed in an electrolyte solution to the level of the junction between glabrous and hairy skin. The volume of fluid displaced was detected by a transducer and the measurement in milliliters displayed on a digital light-emitting

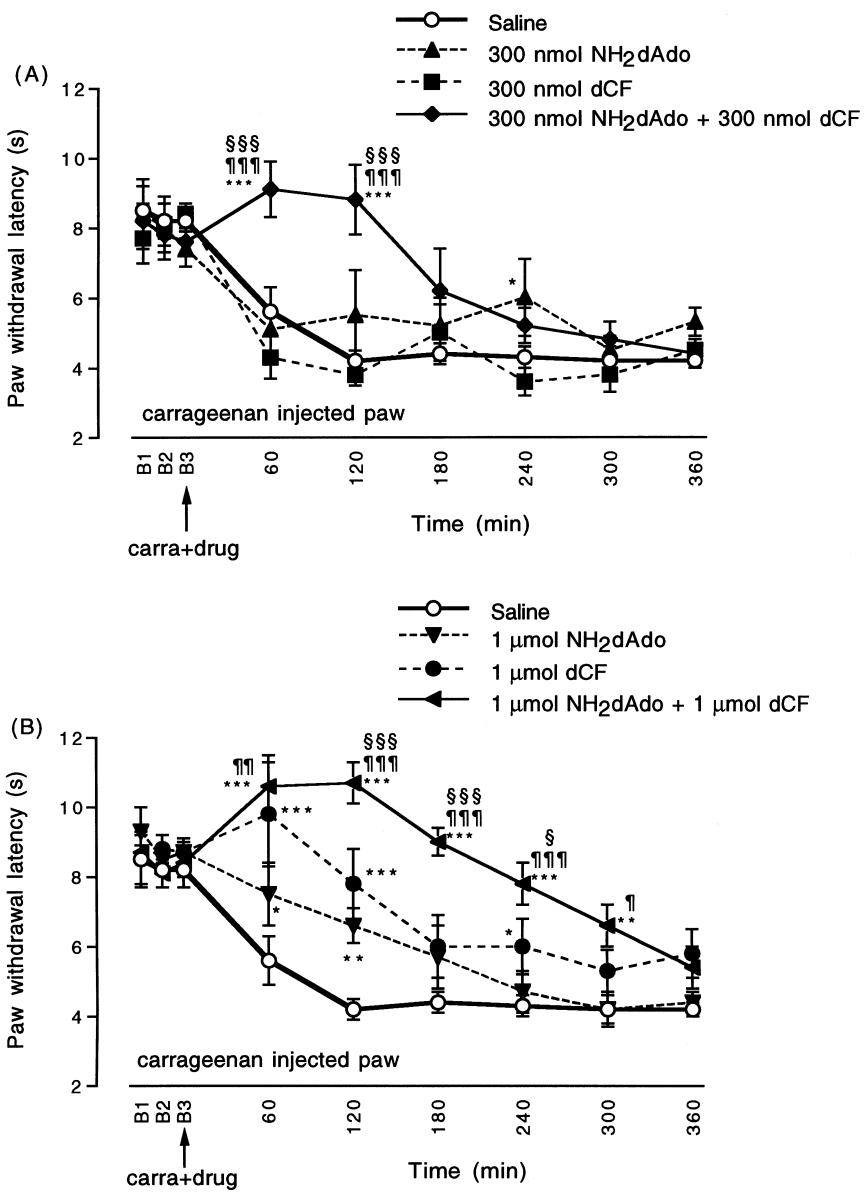


Fig. 1. Time course for antinociceptive effects produced by the *ipsilateral* administration of (A) 300 nmol and (B) 1 μmol 5′-amino-5′-deoxyadenosine (NH_2dAdo), 2′-deoxycoformycin (dCF) and a combination of both agents. Individual drugs and combinations were injected immediately prior to carrageenan (carra) injection. Mean ± S.E.M. values are presented. *, ** and *** denote, respectively, $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. saline control; ¶, ¶¶ and ¶¶¶ represent $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. 5′-amino-5′-deoxyadenosine; § and §§§ indicate $P < 0.05$ and $P < 0.001$ vs. 2′-deoxycoformycin. $n = 6–12$.

diode panel. Measurements were made before and at various time-points after carrageenan and drug administration. Each value represents the mean of three repeated measurements.

2.4. Induction of hindpaw inflammation

Under light halothane anaesthesia, lambda carrageenan dissolved in saline (20 mg/ml) was injected subcutaneously (a volume of 100 μl containing 2 mg carrageenan) into the plantar surface of either hindpaw (chosen randomly). The injected hindpaw became inflamed 2–3 h post-injection, as indicated by the development of oedema, erythema and hyperalgesia to thermal and mechanical stimuli (Winter et al., 1962). Maximum hyperalgesia occurred at 3–6 h after carrageenan injection, and the inflammation was mostly resolved by 24 h (Hylden et al., 1991). Except for a tendency to guard the inflamed paw, carrageenan-injected rats demonstrated normal eating, grooming and exploratory behaviour.

2.5. Intraplantar injection

To examine drug effects at the site of inflammation, the drug or drug combination tested was injected subcutaneously into the plantar surface of the carrageenan-injected (ipsilateral) or non-carrageenan-injected (contralateral) paw, depending on the experimental design. Baseline paw withdrawal latency and paw volume measurements were made before the beginning of an experiment. Drugs were injected immediately prior to the injection of carrageenan, except for one group of experiments in which the drug combination was administered 3 h after carrageenan injection. Injection volumes were 30 μl for agonists and 20 μl for antagonists. After carrageenan and drug injection, paw withdrawal latency and paw volume were measured at hourly intervals for up to 6 h. In experiments where drugs were given 3 h after carrageenan injection, post-drug paw withdrawal latency measurements were made at 20 min intervals for 3 h.

2.6. Intrathecal catheterization and injection

In one experiment, caffeine was injected via a surgically implanted intrathecal catheter (Yaksh and Rudy, 1976). The surgical technique has been described previously (Poon and Sawynok, 1998). Briefly, under halothane anaesthesia, a 7.5-cm segment of an 11-cm PE-10 tubing was inserted into the subarachnoid space through the cisterna magna to the rostral extent of the lumbar enlargement. The catheter was secured to the surrounding muscle by a suture, filled with saline, and the open-end was occluded. Animals showing post-surgical motor deficits were euthanized, and experiments were performed 7–10 days after catheterization (90% success rate of implantation). Upon injection, the rat was briefly accommodated in a restraining box while 10 μl of the drug solution was administered followed by 10 μl of saline to ensure complete drug delivery (cannula volume: 8 μl). Ninety minutes after the administration of the agonists to the ipsilateral paw, caffeine (1545 nmol) was injected intrathecally. Paw withdrawal latency measurements were made at 15 min intervals for 1 h, followed by hourly measurements for 3 h.

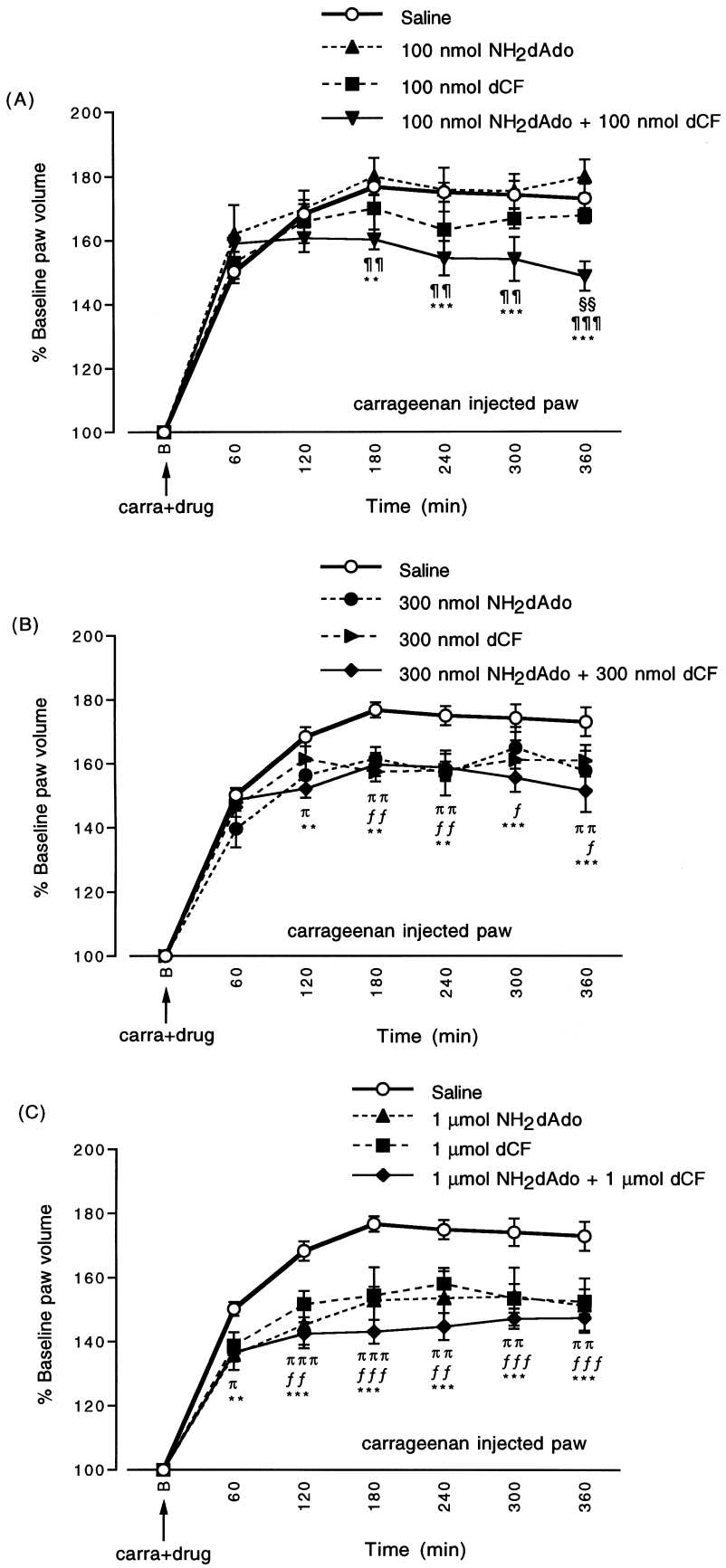
2.7. Measurement of carrageenan-induced spinal c-Fos expression

As in behavioural experiments, hindpaw inflammation was induced by an intraplantar injection of 2 mg/100 μl carrageenan under light halothane anaesthesia. An ipsilateral vehicle or drug injection (30 μl) was made immediately prior to the carrageenan injection. Rats were returned to their cages after the injections.

Three hours after carrageenan injection, the animal was heavily anaesthetized with pentobarbital (55 mg/kg by intraperitoneal injection). After exposing the heart, an opening was made on the left ventricular wall to allow the perfusion catheter access to the ascending aorta. Upon securing the position of the catheter with a pair of hemostatic forceps, buffer flow into the aorta was established, and the right atrium was punctured to allow the outflow of blood and perfusion buffer. The animal was perfused for approximately 15 min with a 0.1% solution of sodium nitrite in 0.05M phosphate-buffered saline (pH 7.4) until the outflow appeared blood-free. This was followed by perfusion with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 30 min. A laminectomy was then performed and the lumbar segment of the spinal cord was removed and stored at 4°C, first in 4% paraformaldehyde/phosphate buffer and subsequently in a 20% sucrose solution, for 48 and 24 h, respectively.

Using a freezing microtome (American Optical, NY), 40 μm sections were cut from the L_4–L_5 segments. Free-floating sections were immunostained for c-Fos-like

Fig. 2. Time course for effects on paw swelling by the *ipsilateral* administration of (A) 100 nmol, (B) 300 nmol and (C) 1 μmol 5′-amino-5′-deoxyadenosine (NH_2dAdo), 2′-deoxycoformycin (dCF) and a combination of both agents. Individual drugs and combinations were injected immediately prior to carrageenan (carra) injection. Paw volume measurements from the ipsilateral paw are presented as percentages of corresponding baseline paw volumes. Values are mean ± S.E.M. ** and *** denote, respectively, $P < 0.01$ and $P < 0.001$ for the combination group vs. saline control; ¶¶ and ¶¶¶ represent $P < 0.01$ and $P < 0.001$ vs. 100 nmol 5′-amino-5′-deoxyadenosine; §§ indicates $P < 0.01$ vs. 100 nmol 2′-deoxycoformycin; π, ππ and πππ denote $P < 0.05$, $P < 0.01$ and $P < 0.001$ for 5′-amino-5′-deoxyadenosine vs. saline; *f*, *ff* and *fff* represent $P < 0.05$, $P < 0.01$ and $P < 0.001$ for 2′-deoxycoformycin vs. saline. $n = 6–11$.


(A)
Saline
100 nmol NH2dAdo
100 nmol dCF
100 nmol NH2dAdo + 100 nmol dCF
% Baseline paw volume
carrageenan injected paw
carra+drug
Time (min)
(B)
Saline
300 nmol NH2dAdo
300 nmol dCF
300 nmol NH2dAdo + 300 nmol dCF
% Baseline paw volume
carrageenan injected paw
carra+drug
Time (min)
(C)
Saline
1 μmol NH2dAdo
1 μmol dCF
1 μmol NH2dAdo + 1 μmol dCF
% Baseline paw volume
carrageenan injected paw
carra+drug
Time (min)

protein employing the avidin–biotin–peroxidase complex method. A sheep polyclonal immunoglobulin G (IgG) antibody to *fos* oncoproteins (Genosys) was used as the primary antibody (1:1000 dilution), whereas the secondary antibody used was a biotinylated rabbit anti-sheep IgG antibody (Vector Laboratories) in a 1:500 dilution. Immunostained Fos protein was made visible using a chromagen, diaminobenzidine (Sigma, St. Louis, MO), the colour intensity of which was enhanced by the addition of ammonium nickel sulphate. Stained sections were arranged by spinal segment based on the gross morphology of the grey matter, mounted on subbed microscope slides (VWR Canlab) and cover-slipped using Entellan (BDH) as the mounting medium.

Twenty sections from the L_4–L_5 segment were selected at random. Fos-labeled neurons, regardless of the intensity of staining, were counted visually under light microscopy (Olympus BH-2). Accuracy of counting was verified using a computerized imaging system. Briefly, the section was captured as an image file using a video camera (JVC) attached to the microscope and connected to an Apple® Centris 650 running the Adobe Photoshop® software. Using the NIH Image software, the area to be counted was selected and Fos-like immunoreactivity counted as clusters of pixels. The number of clusters indicates the number of Fos-positive nuclei.

To study the laminar distribution of Fos, the dorsal horn was divided into: superficial dorsal horn (laminae I–II),

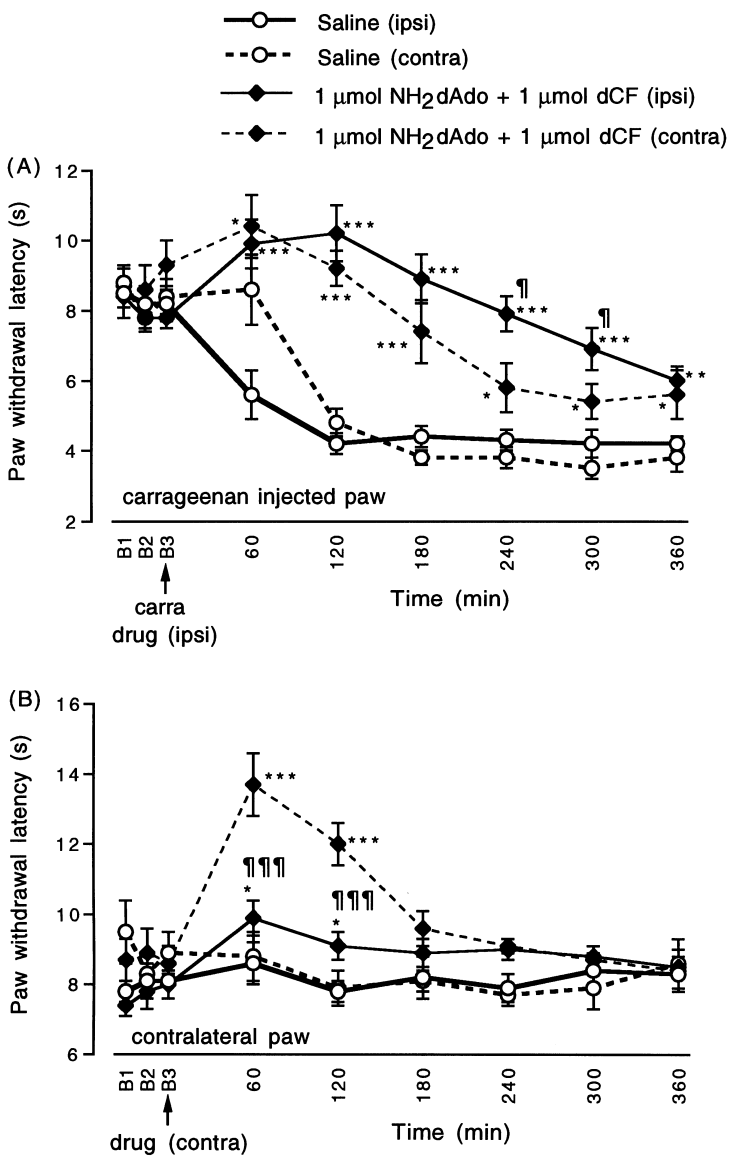


Fig. 3. Comparison of antinociceptive effects produced by an *ipsilateral* (ipsi) vs. a *contralateral* (contra) co-administration of 1 μmol 5′-amino-5′-deoxyadenosine (NH_2dAdo) and 1 μmol 2′-deoxycoformycin (dCF). Values are mean ± S.E.M. *, ** and *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to the corresponding saline group; ¶ and ¶¶¶ indicate $P < 0.05$ and $P < 0.001$ compared to 1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin (contra). $n = 7$–11.

nucleus proprius (laminae III–IV) and neck of the dorsal horn (laminae V–VI). The total number of Fos-positive neurons (per region and all regions) in 20 sections was recorded and analysed for statistical difference.

2.8. Drugs

5′-Amino-5′-deoxyadenosine, caffeine and lambda carrageenan were purchased from Sigma; 2′-deoxycoformycin was a gift from Parke-Davis Pharmaceutical Research Division, Warner Lambert, Ann Arbor, MI; 3,7-dimethyl-1-propargylxanthine (DMPX) was purchased from Research Biochemicals, Natick, MA. Drugs and colloidal suspensions of carrageenan were prepared fresh for each experiment. All agents were dissolved in normal saline.

2.9. Statistical analysis

In behavioural experiments, statistical significance was established by repeated measures analysis of variance (RM ANOVA) followed by the Least Significant Difference (LSD) post-hoc test. Statistical significance in c-Fos experiments was established by ANOVA followed by the LSD post-hoc analysis. Levels of significance were set at 0.05, 0.01 and 0.001.

3. Results

3.1. Antinociceptive and anti-inflammatory effects of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin

Injection of 2 mg/100 μl carrageenan into the hindpaw led to a reduction in withdrawal latency from a heat source as well as significant paw swelling. Paw withdrawal latencies were found to be significantly reduced compared to baseline values, starting at 2 h and lasting for up to 6 h (Fig. 1, saline group), whereas a significant increase in paw volume was seen at all time points throughout the experiment (Fig. 2, saline group).

5′-Amino-5′-deoxyadenosine and 2′-deoxycoformycin were injected at doses of 100 nmol, 300 nmol and 1 μmol. The drug was injected into the paw immediately prior to the injection of carrageenan. At 100 nmol ($n = 6$; data not shown) and 300 nmol (Fig. 1A), neither 5′-amino-5′-deoxyadenosine nor 2′-deoxycoformycin demonstrated antinociceptive properties in the carrageenan-injected (ipsilateral) paw. A modest yet significant reduction in the rate of development of thermal hyperalgesia in the ipsilateral paw was produced by 1 μmol 5′-amino-5′-deoxyadenosine (Fig. 1B); this also produced a mild analgesic effect in the contralateral paw at 60 min (change in withdrawal latency: 2.08 ± 0.52 s; $P < 0.05$) and 120 min (change in withdrawal latency: 1.94 ± 0.71 s; $P < 0.05$). 2′-Deoxycoformycin 1 μmol also produced significant antihyperalgesia at 60 min and 120 min in the ipsilateral paw (Fig. 1B), as well as a mild analgesia in the contralateral paw (change in withdrawal latency: 1.53 ± 0.44 s at 60 min and 2.11 ± 0.46 s at 120 min; $P < 0.05$ for both times). An analgesic effect seen in the contralateral paw suggests that drug actions are mediated via systemic absorption and redistribution.

At 100 nmol, neither 5′-amino-5′-deoxyadenosine nor 2′-deoxycoformycin given alone reduced paw swelling (Fig. 2A). Despite the lack of effect on nociceptive thresholds, 300 nmol 5′-amino-5′-deoxyadenosine and 2′-de-

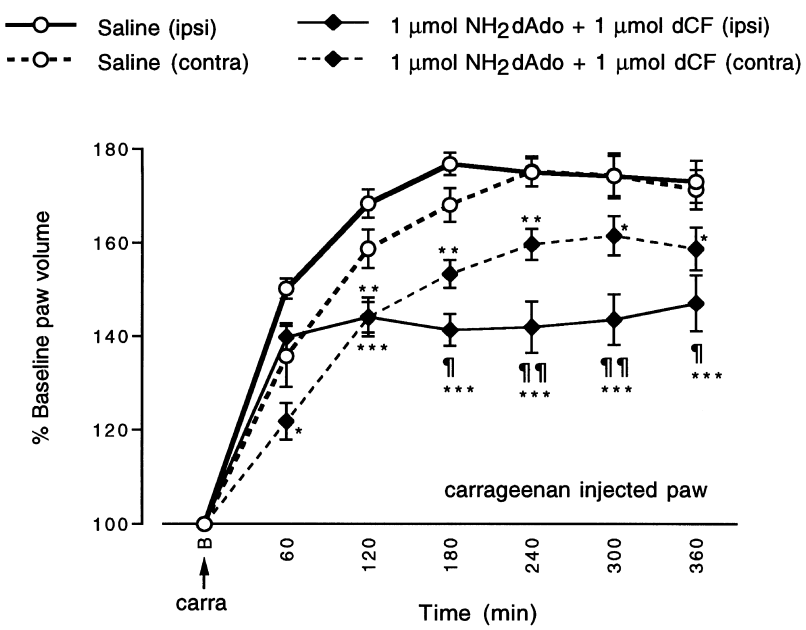


Fig. 4. Comparison of effects on paw swelling by *ipsilateral* (ipsi) vs. *contralateral* (contra) co-injections of 1 μmol 5′-amino-5′-deoxyadenosine (NH_2dAdo) and 1 μmol 2′-deoxycoformycin (dCF). Values are mean ± S.E.M. *, ** and *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to the corresponding saline group; ¶ and ¶¶ denote $P < 0.05$ and $P < 0.01$ compared to the contralateral (contra) injection group. $n = 5–11$.

oxycoformycin given alone produced a modest reduction in paw swelling 3–6 h after carrageenan injection (Fig. 2B). At 1 μmol, both agents significantly reduced paw oedema throughout the entire test period; effects were seen as early as 1 h post-carrageenan (Fig. 2C).

3.2. Antinociceptive and anti-inflammatory effects of combinations of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin

To investigate the interactions between inhibitors, various dosage combinations of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin were administered and their effects compared to those elicited by the individual agents. No antinociception was seen with a combination of 100 nmol 5′-amino-5′-deoxyadenosine + 100 nmol 2′-deoxycoformycin ($n = 6$; data not shown). When co-injected, 5′-amino-5′-deoxyadenosine 300 nmol + 2′-deoxycoformycin 300 nmol produced significant antihyperalgesia in the carrageenan-injected paw, delaying the development of thermal hyperalgesia for 2 h (Fig. 1A). The antinociceptive effects of 1 μmol 5′-amino-5′-deoxyadenosine and 1 μmol 2′-deoxycoformycin were enhanced both in terms of magnitude and duration when the two drugs were co-administered (Fig. 1B). Ipsilateral 1 μmol 5′-amino-5′-deoxy-

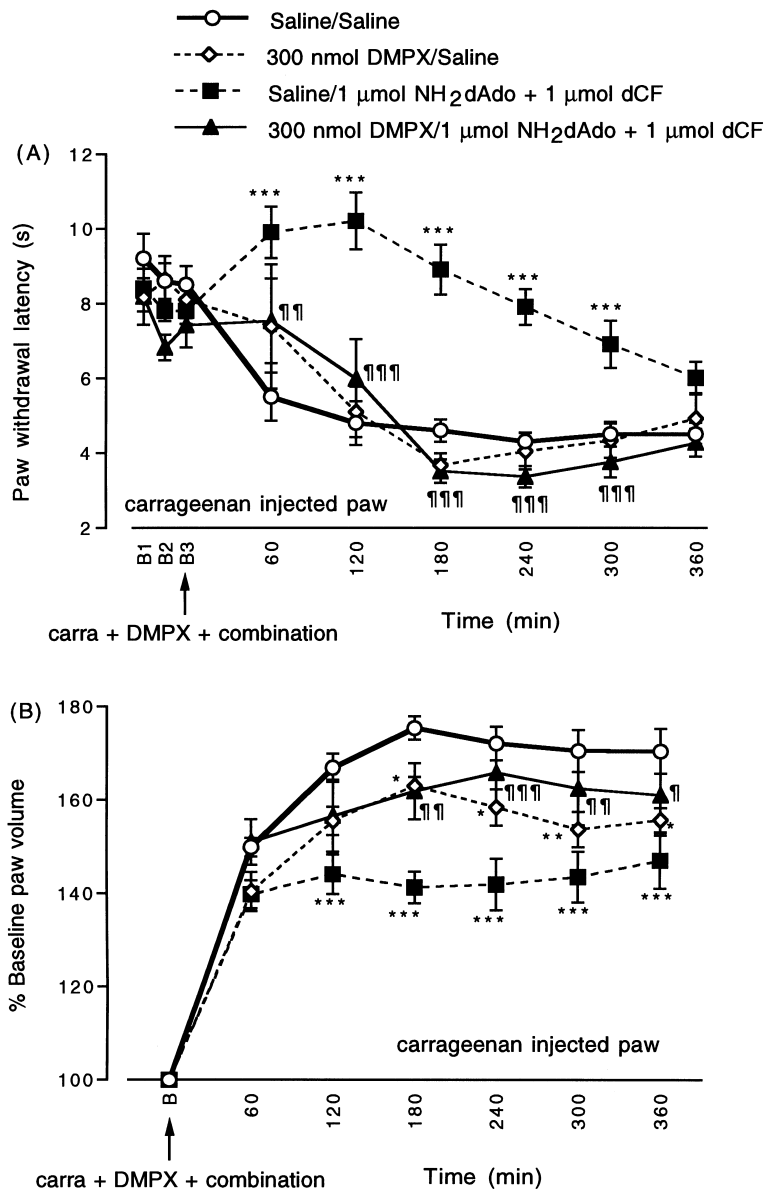


Fig. 5. Antagonistic effect of *ipsilaterally* administered DMPX (300 nmol) against (A) antinociception and (B) paw swelling reduction produced by the *ipsilateral* co-administration of 1 μmol 5′-amino-5′-deoxyadenosine (NH_2dAdo) and 1 μmol 2′-deoxycoformycin (dCF). The antagonist was injected immediately prior to the administration of the drug combination, which was followed immediately by the carrageenan (carra) injection. Mean ± S.E.M. values are presented. *, ** and *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. the saline/saline group; ¶, ¶¶ and ¶¶¶ denote $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. the saline/1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin group. $n = 5–12$.

adenosine + 1 μmol 2′-deoxycoformycin also produced a slight antinociception in the contralateral paw at 60 and 120 min (Fig. 3B), suggesting a systemic component of activity.

Measurement of paw volumes in the same experiment revealed that 100 nmol 5′-amino-5′-deoxyadenosine + 100 nmol 2′-deoxycoformycin, which had no antinociceptive action, reduced paw swelling (Fig. 2A). The initial rate of development of oedema was not altered, and the paw volume reduction was seen only at later time points (180 min and beyond). The amount of reduction in paw swelling produced by the 100 nmol combination was comparable to that produced by the 300 nmol combination (cf. Fig. 2A and B), whereas co-administration of 1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin reduced paw swelling to a greater extent, amounting to more than 30% reduction (Fig. 2C). Despite an augmentation in antinociception (cf. Fig. 1A and B), no significant enhancement in the reduction of paw swelling was evident with the 300 nmol or the 1 μmol combinations of 5′-amino-5′-deoxyadenosine + 2′-deoxycoformycin (Fig. 2B and C) compared to results of these agents alone.

3.3. Local *vs.* systemic effects of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin combinations

The increases in contralateral paw withdrawal latency produced by an ipsilateral drug injection indicate that

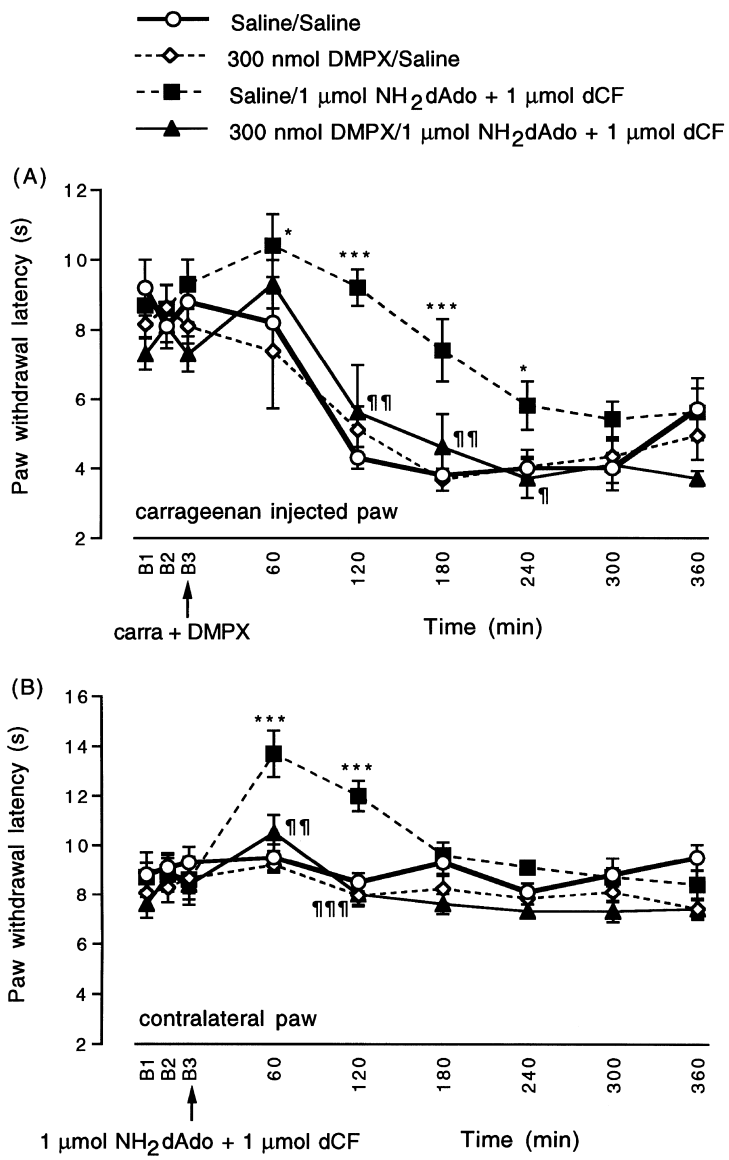


Fig. 6. Antagonistic effect of *ipsilaterally* administered DMPX (300 nmol) against antinociception produced by the *contralateral* co-administration of 1 μmol 5′-amino-5′-deoxyadenosine (NH_2dAdo) and 1 μmol 2′-deoxycoformycin (dCF). Drug injections were made in immediate succession. Mean ± S.E.M. values are presented. * and *** represent $P < 0.05$ and $P < 0.001$ vs. the saline/saline group; ¶, ¶¶ and ¶¶¶ indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. the saline/1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin group. $n = 4–10$.

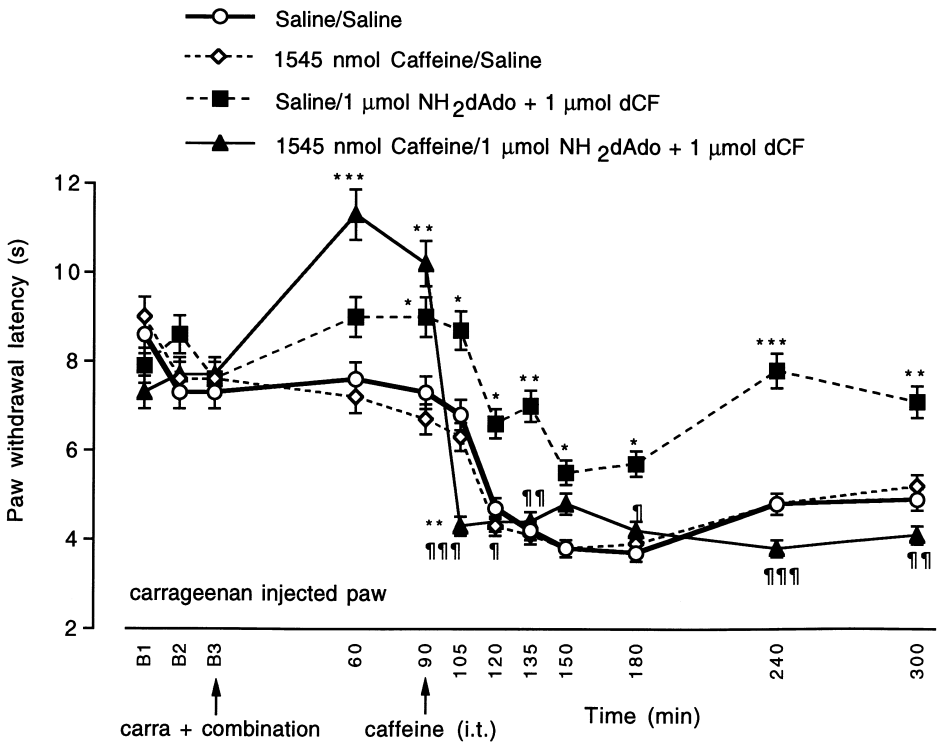


Fig. 7. Antagonistic effect of *intrathecally* (i.t.) administered caffeine (1545 nmol) against antinociception produced by the *ipsilateral* co-administration of 1 μmol 5′-amino-5′-deoxyadenosine (NH_2dAdo) and 1 μmol 2′-deoxycoformycin (dCF). The antagonist was administered via an implanted catheter 90 min after the injection of the drug combination and carrageenan (carra). Mean ± S.E.M. values are presented. *, ** and *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. the saline/saline group; ¶, ¶¶ and ¶¶¶ indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. the saline/1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin group. $n = 4–6$.

systemic drug actions occur at higher doses of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin. Further experiments were conducted in an attempt to distinguish local from systemic effects, whereby combinations of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin (100 nmol, 300 nmol and 1 μmol of each drug) were injected into the contralateral (non-carrageenan-injected) paw, followed by measurements of paw withdrawal latency and paw volume for both paws. The 1 μmol combination produced pronounced antinociceptive effects that lasted for 120–180 min, both in the carrageenan-injected (Fig. 3A) and the contralateral paw (Fig. 3B). Antihyperalgesia of a lesser magnitude and shorter duration was seen with the 300 nmol combination in the carrageenan-injected paw and in the contralateral paw (data not shown). When paw withdrawal latency data from ipsilateral and contralateral groups were compared, it was noted that ipsilateral drug injections produced antihyperalgesia of a longer duration in the carrageenan-injected paw than when the drugs were injected contralaterally. However, the maximum paw withdrawal latencies were not different between the groups (Fig. 3A). Administration of 1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin to the contralateral paw resulted in marked antinociception in the same paw 60 and 120 min after drug injection (Fig. 3B), and this was greater than that seen following injection into the opposite paw. The different time–response relationships

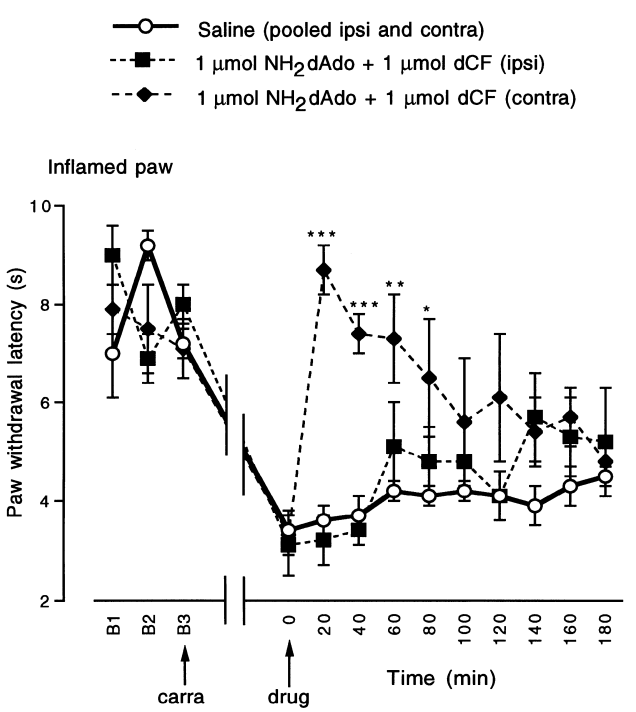


Fig. 8. Comparison of antihyperalgesia in the inflamed paw produced by an *ipsilateral* (ipsi) vs. a *contralateral* (contra) co-administration of 1 μmol 5′-amino-5′-deoxyadenosine (NH_2dAdo) and 1 μmol 2′-deoxycoformycin (dCF) 3 h after the induction of inflammation by carrageenan. Values are mean ± S.E.M. *, ** and *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to the saline group. $n = 4–6$.

produced by ipsilateral vs. contralateral drug injections provide evidence for both local and systemic antinociceptive activity, although the latter effect predominates.

Contralateral injection of the higher-dose combination also reduced swelling of the carrageenan-injected paw, albeit to a lesser extent than with the ipsilateral injection. Thus, *contralateral* injection of the 1 μmol combination reduced paw oedema by approximately 15%, compared to a reduction of more than 30% by the *ipsilateral* injection (Fig. 4). Significant differences between the ipsilateral and contralateral groups were found at various time points. The reduction of paw swelling by a contralateral injection suggested a systemic drug action when high dose combinations of the adenosine kinase inhibitor and the adenosine deaminase inhibitor were administered. A *contralateral* co-injection of 300 nmol 5′-amino-5′-deoxyadenosine + 300 nmol 2′-deoxycoformycin failed to significantly reduce paw swelling ($n = 6$; data not shown), while the same regimen administered *ipsilaterally* reduced paw swelling by 15%–18% (Fig. 2B).

3.4. Antagonism of effects produced by a combination of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin by an adenosine receptor antagonist

To determine if the adenosine A_{2A} receptor was involved in the anti-inflammatory effects produced by the peripheral injection of a combination of 1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin, DMPX, a somewhat selective A_{2A} antagonist, was used. When injected with the adenosine kinase and deaminase inhibitor combination, 300 nmol DMPX completely antagonized the antihyperalgesic effect (Fig. 5A), but only partially reduced paw swelling induced by the drug combination (Fig. 5B). DMPX, 100 nmol, failed to block the effects of the 1 μmol drug combination (data not shown). When the agonists were administered to the *contralateral* paw, antinociception was seen in both paws, and the effects were blocked by *ipsilateral* DMPX (Fig. 6). Besides antagonizing antinociceptive effects, *ipsilateral* DMPX also reversed the paw volume reduction induced by the *contralat-*

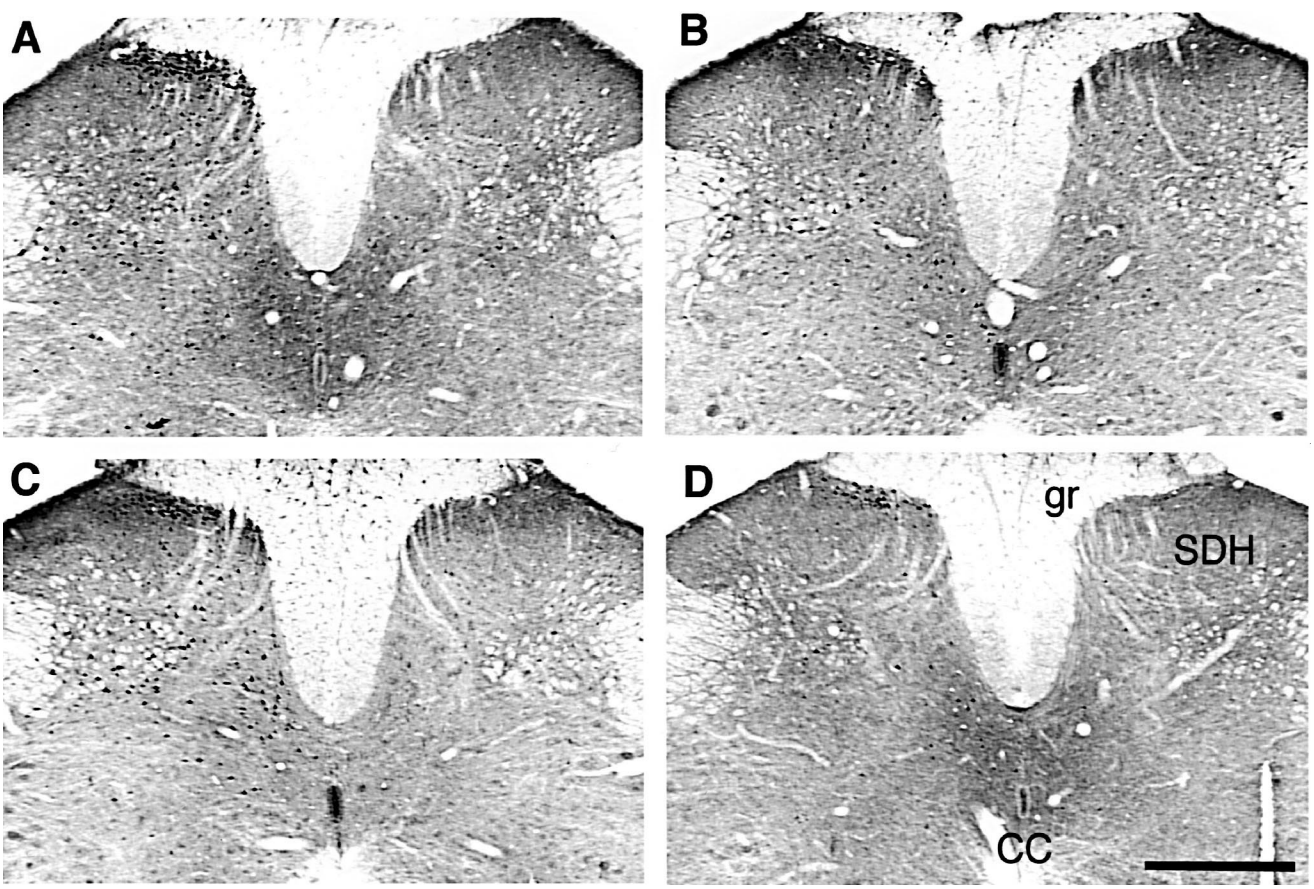


Fig. 9. Photomicrographs showing the effects of various treatments on spinal c-Fos expression 3 h after intraplantar carrageenan injection. Drugs were administered immediately prior to the injection of carrageenan (carra). Presented are representative sections from four treatment groups: (A) saline, (B) 1 μmol 5′-amino-5′-deoxyadenosine (NH_2dAdo), (C) 1 μmol 2′-deoxycoformycin (dCF), and (D) 1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin combination. cc = central canal; gr = gracile fasciculus; SDH = spinal dorsal horn. Scale bar: 200 μm.

eral 1 μmol drug combination (data not shown). Reversal of both antinociception and paw volume effects in the contralateral paw by an *ipsilateral* injection of DMPX (cf. Fig. 6B) indicated systemic activity of the antagonist.

3.5. Reversal of antihyperalgesia by spinally administered caffeine

It became apparent that antinociception produced by the 1 μmol combination administered peripherally was mediated, at least in part, through a systemic drug action. In order to examine the possibility of a spinal site of action, caffeine was injected intrathecally via an implanted catheter to reverse antinociception produced by an ipsilaterally injected combination of 1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin. Caffeine (1.55 μmol) was administered 90 min after the carrageenan and ipsilateral drug injection, and produced a complete reversal of the antihyperalgesia in the inflamed paw which lasted throughout the entire test period (Fig. 7). The time for caffeine injection was chosen to coincide with the period during which maximum agonist effects were evident.

3.6. Effects of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin combinations on established hyperalgesia

While combinations of 5′-amino-5′-deoxyadenosine + 2′-deoxycoformycin retarded the development of thermal hyperalgesia and paw swelling induced by intraplantar carrageenan injection, it was not clear whether drug regimens could reverse an established hyperalgesia and paw oedema. Hence, drug combinations were injected ipsilaterally or contralaterally 3 h after the injection of carrageenan, a time by which significant hyperalgesia and paw swelling were established. Interestingly, *contralateral*, but not an ipsilateral, administration of the 1 μmol combination significantly reversed hyperalgesia (Fig. 8). Antinociception peaked at 20 min post-injection and lasted for more than 60 min. No reduction in paw swelling was seen with administration of the drug regimen to either paw (data not shown).

3.7. Reduction of carrageenan-induced spinal c-Fos expression by peripheral injection of a 5′-amino-5′-deoxyadenosine + 2′-deoxycoformycin combination

Injection of 2 mg/100 μl carrageenan increased spinal levels of the immediate-early gene product c-Fos detected by immunohistochemical staining. Three hours after carrageenan injection, Fos-like immunoreactivity was seen predominantly in the ipsilateral spinal dorsal horn of sections from the lumbar (L_4–L_5 segment) spinal cord (Figs. 9A and 10). Increases in Fos-like immunoreactivity were found in various laminae of the dorsal horn, with the highest density observed in laminae I and II (Figs. 9 and 10). Intraplantar administration of saline or a combination

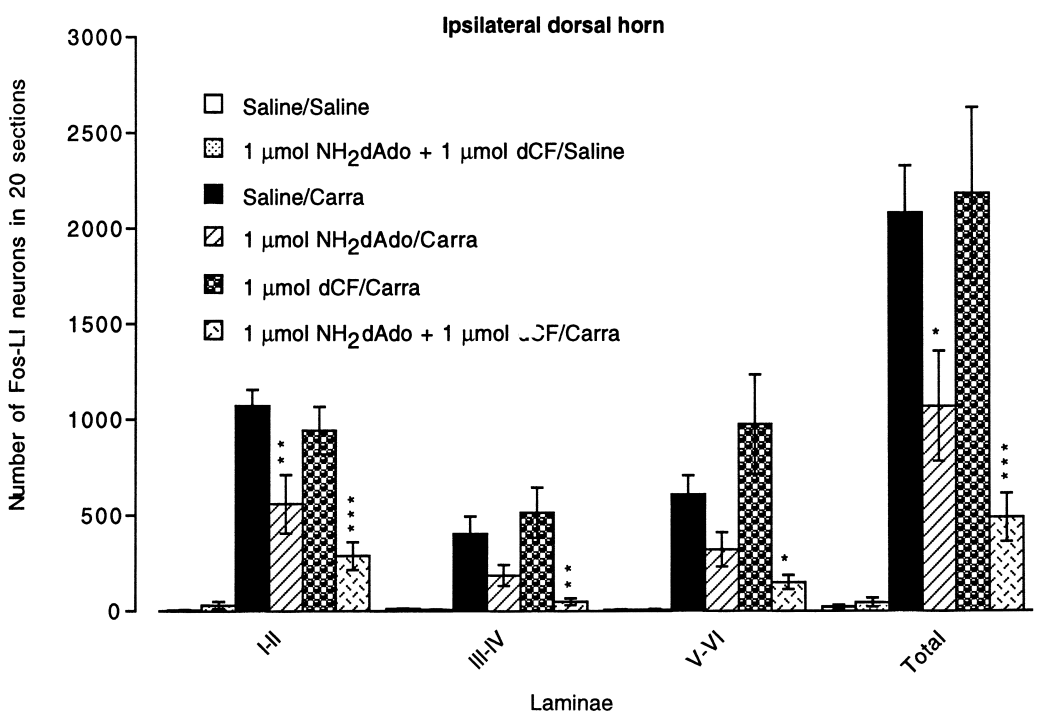


Fig. 10. Effects of drug treatment on the expression of c-Fos in the spinal dorsal horn 3 h after intraplantar carrageenan (carra) and drug injection. Twenty sections from the L_4–L_5 segments were selected randomly; the total number of Fos-like immunoreactivity in 20 sections and their laminar distribution was recorded and analyzed. Values are mean ± S.E.M. *, ** and *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$ compared to the saline/carra group. $n = 4$–6 per group.

of 1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin, without a simultaneous carrageenan injection, did not result in a unilateral increase in Fos expression (Fig. 10).

Ipsilateral injection of 1 μmol 5′-amino-5′-deoxyadenosine significantly reduced Fos expression in the dorsal horn by approximately 50%. A general reduction in Fos was seen in all laminae; statistical significance was, however, established only in the analyses of laminae I–II as well as total Fos counts (Fig. 10). Despite a lack of effect with the same dose of 2′-deoxycoformycin, a greater reduction (~75%) in Fos-like immunoreactivity was evident in all laminae when 1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin was administered to the carrageenan-injected paw (Fig. 10). However, ANOVA showed no significant difference between the effect of the drug combination and 1 μmol 5′-amino-5′-deoxyadenosine by itself.

4. Discussion

Increasing evidence supports a role for adenosine in peripheral nociceptive processing (reviewed in Sawynok, 1998). In the present experiment, the possibility of a peripheral antinociceptive action of inhibitors of adenosine kinase and adenosine deaminase was examined using the carrageenan model. The peripheral modulation of nociceptive signaling in this test has only received limited attention to date (see Jackson et al., 1995), although a number of recent studies have revealed peripheral modulation of pain in the formalin test by various agents known primarily for their central actions, such as excitatory amino acid receptor antagonists (Davidson and Carlton, 1998), anticonvulsants (Carlton and Zhou, 1998), antidepressants (Sawynok et al., 1999), and adenosine kinase and adenosine deaminase inhibitors (Sawynok et al., 1998). Adenosine produces a diverse range of effects on the inflammatory process by acting on inflammatory cells, and may be an endogenous anti-inflammatory agent (reviewed in Cronstein, 1997). The present study examined the effectiveness of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin, either alone or in combination, in reversing carrageenan-induced thermal hyperalgesia as well as in retarding the development of paw oedema when injected immediately prior to carrageenan.

4.1. Activity of an adenosine kinase inhibitor and an adenosine deaminase inhibitor against pain and inflammation

In contrast to the marked antinociception seen after spinal administration (Poon and Sawynok, 1998), 300 nmol 5′-amino-5′-deoxyadenosine, when injected into the hindpaw immediately prior to carrageenan, did not produce any antinociception. A higher dose (1 μmol) produced only a mild antihyperalgesia and analgesia in the ipsilateral and contralateral paw, respectively. Activity in the contralateral paw suggested a systemic drug action. Interestingly, both doses significantly reduced paw swelling by 15%–20% for the entire 6-h test period. There is thus a dissociation between the antihyperalgesic and anti-inflammatory effects produced by the adenosine kinase inhibitor. A similar separation is seen with 2′-deoxycoformycin, where paw oedema, but not thermal hyperalgesia, is reduced at a dose of 300 nmol. These results indicate that (a) although inflammation and hyperalgesia are closely inter-related, the two processes may be differentially modulated and (b) attenuating inflammation does not necessarily result in a reversal of hyperalgesia.

While spinal administration of an adenosine deaminase inhibitor failed to produce antinociception (Poon and Sawynok, 1998), adenosine deaminase inhibition in the periphery produced antinociceptive and anti-inflammatory effects to a comparable extent as those produced by the inhibition of adenosine kinase. This may reflect a more pronounced elevation in endogenous adenosine levels as a result of inhibition of paw tissue adenosine deaminase. During acute inflammation, there is an increase in blood flow into the affected area, followed by congestion and stasis of blood cells in capillaries. High adenosine levels, as well as adenosine deaminase activity, has been demonstrated in blood cells, especially erythrocytes (see Geiger et al., 1991). Thus, abundant erythrocytes in inflamed tissue could constitute a rich source of adenosine, and adenosine deaminase likely plays an important role in regulating its level. Inhibition of adenosine deaminase by 2′-deoxycoformycin could, therefore, lead to a marked increase in adenosine concentration in the inflamed tissue, and activation of adenosine A_1 receptors on primary nociceptive afferents, as well as adenosine A_{2A} receptors on inflammatory cells resulting in antinociception and a reduction in paw swelling, respectively.

4.2. Enhancement of antinociception and anti-inflammatory effects by an adenosine kinase inhibitor and an adenosine deaminase inhibitor in combination

Simultaneous inhibition of peripheral adenosine kinase and adenosine deaminase results in a marked enhancement of antinociceptive activity, but only a minimal enhancement of anti-inflammatory effects. At 100 nmol, neither 5′-amino-5′-deoxyadenosine nor 2′-deoxycoformycin alone demonstrated antinociceptive or anti-inflammatory properties. While co-administration of both agents significantly reduced paw oedema, the regimen did not influence thermal hyperalgesia. A supra-additive interaction occurred between 300 nmol 5′-amino-5′-deoxyadenosine and 300 nmol 2′-deoxycoformycin against thermal thresholds, but the combination did not produce an enhanced anti-inflammatory effect. Both antinociceptive and anti-inflammatory effects produced by 1 μmol each of 5′-amino-

5′-deoxyadenosine and 2′-deoxycoformycin were enhanced when the two drugs were administered in combination (antinociception > reduction in paw oedema). The interaction between these two agents is less dramatic than when the drugs are administered intrathecally (see Poon and Sawynok, 1998). This is likely due to a differential contribution of adenosine kinase and adenosine deaminase to regulating adenosine levels at peripheral sites compared to the dorsal spinal cord.

4.3. Local vs. systemic effects of the intraplantar administration of inhibitors of adenosine kinase and adenosine deaminase

One of the objectives of these experiments was to determine whether manipulating endogenous adenosine levels in inflamed tissue results in peripherally mediated antinociception, presumably via activation of adenosine receptors on primary nociceptive afferents. Thus, there is evidence to support the presence of adenosine receptors on sensory afferent nerve terminals, the activation of which produces antinociception (see Section 1). To differentiate between local and systemic effects, results from animals receiving an ipsilateral or contralateral injection of the same dose regimen were compared. Drug effects in the non-injected paw indicate systemic drug activity. While evidence for local drug actions against thermal thresholds was observed, this component seems mild and may play only a secondary role in antinociception produced by inhibitors of adenosine kinase and adenosine deaminase in this test. No drug or drug combination produced an exclusively peripheral effect on hyperalgesia, as all dose regimens that were effective when injected ipsilaterally also demonstrated mild antinociceptive properties in the contralateral paw. Furthermore, combinations of higher doses of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin injected into the contralateral paw also produced significant antihyperalgesia in the carrageenan-injected paw. Finally, significant antinociception in the inflamed paw was seen when 1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin was administered into the contralateral paw 3 h following carrageenan, whereas the combination was ineffective when directly injected into the hindpaw with established inflammation and hyperalgesia. The total lack of effect in the latter case not only confirms a lack of local drug activity, but also reveals a possible impairment of drug absorption in inflamed tissue. Impaired drug absorption is most likely due to a congestion of capillaries with blood cells, as well as the continuous exudation of fluids.

The 1 μmol combination most likely produces antinociception via activation of spinal adenosine receptors, as drug effects are reversed almost instantaneously by spinally administered caffeine. Thus, it is likely that after intraplantar administration, inhibitors of adenosine kinase and adenosine deaminase are absorbed and transported to the dorsal spinal cord, where inhibition of adenosine kinase and adenosine deaminase elevates endogenous adenosine levels (cf. Golembiowska et al., 1995, 1996) to produce antinociception via activation of, most likely, adenosine A_1 receptors.

In contrast to the spinal site of action for antinociception, there is little doubt that the reduction in paw oedema induced by lower-dose combinations of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin is peripherally mediated. Thus, anti-inflammatory effects are seen with ipsilateral, but not contralateral, injections of 100 and 300 nmol combinations of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin. At the 1 μmol dose, co-injection of the two agents into the contralateral paw did reduce paw swelling, but the effect was less pronounced with an ipsilateral injection. Administered to the contralateral paw, the drugs may be absorbed and carried via the bloodstream to the inflamed paw, where they exert their anti-inflammatory effects by increasing adenosine levels in the paw tissue. In this case, the ''systemically mediated effect'' differs from that noted above for antinociception as it still occurs in a peripheral compartment rather than a central one.

4.4. Involvement of adenosine receptors in antinociception and anti-inflammatory activity

It has been suggested that the anti-inflammatory effect of adenosine is mediated via activation of adenosine A_{2A} receptors on inflammatory cells such as neutrophils (reviewed in Cronstein, 1997). To determine whether adenosine A_{2A} receptors are involved in the anti-inflammatory effects produced by a combination of 1 μmol 5′-amino-5′-deoxyadenosine + 1 μmol 2′-deoxycoformycin, attempts were made to reverse drug effects by DMPX, a somewhat selective adenosine A_{2A} receptor antagonist (Jacobson and van Rhee, 1997). DMPX reverses the reduction in neutrophil accumulation and vascular leakage produced by an adenosine kinase inhibitor (Rosengren et al., 1995). At doses of 30 and 100 nmol, DMPX injected ipsilaterally failed to block antinociception or the reduction in paw swelling induced by an ipsilateral injection of the drug combination. Increasing the dose of DMPX to 300 nmol completely antagonized both the antinociceptive and anti-inflammatory effects of the combination. Surprisingly, ipsilateral administration of the same dose of DMPX also completely reversed antinociception and anti-inflammatory effects induced by a contralateral injection of the agonist combination. These results indicate that DMPX, at the dose tested, not only blocks the peripheral anti-inflammatory effect but also reverses systemic (likely spinal) antinociception produced by the peripheral co-administration of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin. The selectivity of DMPX for the adenosine A_{2A} receptor over the A_1 receptor has been shown to be limited (10–24 fold) (Ukena et al., 1986). Hence, it is likely that DMPX was taken up into the systemic circulation after

intraplantar injection and reversed 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin-induced systemic antinociception by antagonizing spinal adenosine A_1 receptors. The use of more selective adenosine receptor antagonists (e.g., DPCPX and CPT for the A_1 receptor; CGS15943 and KF17837 for the A_{2A} receptor) (see Jacobson and van Rhee, 1997) as well as exclusively locally active doses of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin may be required to further differentiate the receptor subtypes involved in the antinociceptive and anti-inflammatory effects resulting from inhibition of adenosine kinase and adenosine deaminase.

4.5. Effects of intraplantar adenosine kinase and adenosine deaminase inhibitors on carrageenan-evoked c-Fos expression in the spinal dorsal horn

The present study demonstrates that peripherally administered 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin suppress carrageenan-induced c-Fos protein expression in the spinal dorsal horn. Intraplantar injection of 2 mg/100 μl carrageenan induces a significant increase in Fos-like immunoreactivity in the ipsilateral dorsal horn 3 h after carrageenan injection. Fos-like immunoreactivity is visualized mainly in the superficial laminae (laminae I–II) and in the neck of the dorsal horn (laminae V–VI). This is in accordance with findings from previous studies (Draisci and Iadarola, 1989; Noguchi et al., 1991; Honoré et al., 1995a). As well, the laminar distribution of c-Fos expression corresponds to areas in which a high percentage of neurons is activated by noxious stimuli (Willis and Coggeshall, 1991). Since the majority of small-diameter myelinated and unmyelinated afferents terminate in laminae I and II, Fos-like-immunoreactivity-positive neurons found in the superficial dorsal horn may be projection neurons or interneurons driven by primary nociceptive afferents. This is also likely to be true for Fos-like-immunoreactivity-positive neurons in the neck of the dorsal horn, as these deeper neurons receive direct input from nociceptive afferents as well as indirect input from the superficial laminae. In recent years, c-Fos expression has been widely used as a marker in studying the effects of classical and putative analgesic compounds (Abbadie and Besson, 1993; Honoré et al., 1995b, 1996; Buritova et al., 1996a,b). In the present experiments, 1 μmol 5′-amino-5′-deoxyadenosine, when injected simultaneously with carrageenan, reduced Fos-like immunoreactivity in laminae I–II as well as total Fos-like immunoreactivity in laminae I–VI. Reduction in Fos-like immunoreactivity was further enhanced when 1 μmol 2′-deoxycoformycin, ineffective by itself, was co-injected with 1 μmol 5′-amino-5′-deoxyadenosine. Thus, the profiles for Fos suppression by 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin appear to be in good accordance with the spinal antinociceptive properties of the two agents. Taken together with results from the behavioral experiments, it is possible that the attenuation in spinal c-Fos expression is the consequence of inhibition of adenosine kinase and adenosine deaminase in the spinal dorsal horn. However, as a reduction in c-Fos staining in the dorsal horn serves only as an indicator for a likely inhibition of nociceptive input at the spinal level, other sites/mechanisms of drug action cannot be ruled out. Elevation in local adenosine levels can activate pre-synaptic adenosine A_1 receptors on primary afferent terminals to inhibit the release of excitatory amino acids and neuropeptides, as well as raise excitatory thresholds in projection neurons or interneurons via post-synaptic A_1 receptor-mediated hyperpolarization (reviewed in Sawynok, 1998). These actions likely lead to changes at the second-messenger level, such as an inhibition of protein kinase C, inositol 1,4,5-trisphosphate and diacylglycerol activities and a reduction in Ca^{2+} influx, and as a result, the expression of c-*fos* and other immediate-early genes and their protein products is attenuated. Further studies need to be carried out to determine if locally active anti-inflammatory doses of 5′-amino-5′-deoxyadenosine and 2′-deoxycoformycin reduce c-Fos expression in the dorsal horn.

5. Conclusions

Together with previous findings (Poon and Sawynok, 1998), the current study demonstrates a potential for the development of adenosine kinase inhibitors, as well as combinations of inhibitors of adenosine kinase and adenosine deaminase, as novel therapeutic measures against inflammatory pain. Compared to directly acting adenosine analogues examined to date, inhibitors of adenosine metabolism possess a wider therapeutic window between effective antinociception and undesirable motor effects. They also have the advantage of producing therapeutic effects at multiple sites. Thus, antinociception at the spinal level could be enhanced by locally mediated anti-inflammatory (via adenosine A_2 receptors) and antinociceptive (via adenosine A_1 receptors) effects at the site of inflammation. In this event, the non-selectivity of adenosine that accumulates endogenously would have an advantage over either a selective A_1 or A_2 receptor agonist as it potentially can produce a broader range of beneficial effects.

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