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Antinociception by adenosine analogs and an adenosine kinase inhibitor: dependence on formalin concentration

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Abstract

Spinal administration of adenosine analogs and an adenosine kinase inhibitor produces antinociception in thermal threshold tests. In the present study, we determined the effects of N^6-cyclohexyladenosine (adenosine A_1 receptor selective), 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethyl-carboxamidoadenosine (CGS-21680) (adenosine A_{2A} receptor selective), and 5′-*N*-ethylcarboxamidoadenosine (NECA) (non-selective), on formalin induced nociceptive responses (flinching/lifting and licking/biting) using two concentrations of formalin (2% and 5%). We also examined the antinociceptive effects of 5′-amino-5′-deoxyadenosine, an adenosine kinase inhibitor, and deoxycoformycin, an adenosine deaminase inhibitor, under these conditions. Adenosine A_1 receptor agonists, but not the A_{2A} selective agent, produced significant antinociception, as did 5′-amino-5′-deoxyadenosine, but not deoxycoformycin. The extent of antinociception produced was greater with the lower stimulus intensity. The effects of NECA and 5′-amino-5′-deoxyadenosine were inhibited by caffeine, indicating the involvement of cell surface adenosine receptors in their actions. We conclude (a) that the adenosine A_1, but not the A_{2A}, receptor is involved in spinally mediated antinociception, (b) that adenosine kinase is more important than adenosine deaminase in regulating endogenous adenosine levels in the spinal cord, and (c) that stimulus intensity is an important determinant of the efficacy of purines in the spinal cord.

Keywords: Formalin test; Adenosine analgesia; Adenosine kinase; Stimulus intensity

1. Introduction

The direct spinal administration of adenosine analogs produces antinociception in behavioural studies using thermal threshold tests such as the tail-flick and hot plate tests (Post, 1984; DeLander and Hopkins, 1987; Sawynok et al., 1986; Fastbom et al., 1990; Sosnowski et al., 1989). Binding studies have demonstrated the presence of both adenosine A_1 and A_2 receptors in the substantia gelatinosa (Geiger et al., 1984; Choca et al., 1988), and both receptor subtypes have been implicated in antinociception. Release of endogenous adenosine may contribute to spinal antinociception by opioids as well as other agents, as methylxanthines reduce spinal antinociception by morphine and other opioids (DeLander and Hopkins, 1986; Sweeney et al., 1987; Keil and DeLander, 1992), as well as 5-hydroxytryptamine and selective related agonists (DeLander and Hopkins, 1987; Sawynok, 1995). These agents have been shown to enhance adenosine release from spinal cord preparations (Sweeney et al., 1987, 1989; Cahill et al., 1993).

While adenosine release from the dorsal spinal cord can be promoted by exogenous agents, there also appears to be an ongoing tonic release of adenosine that can regulate nociceptive thresholds. Thus, the spinal administration of 5′-aminodeoxyadenosine, an adenosine kinase inhibitor, produces antinociception in the tail flick test (Keil and DeLander, 1992) while methylxanthines produce hyperalgesia (Jurna, 1984; Sawynok et al., 1986). A reduction in tail flick latency is not always observed with intrathecal methylxanthines; the distinguishing feature seems to be stimulus intensity, as reduction in latency is observed with longer (Jurna, 1984; Sawynok et al., 1986) but not with shorter baseline latencies (DeLander and Hopkins, 1986).

All of the studies mentioned above employed tests that are based on a phasic stimulus. However, most

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clinical pain is of a tonic, continuous, rather than phasic nature, and it has been argued that the more continuous stimulus presented by the formalin test more closely models human pain conditions (Dubuisson and Dennis, 1977; Abbott et al., 1982). There is evidence indicating that tonic pain is modulated differently from phasic pain generated by a brief stimulus of high intensity (Abbott et al., 1982; Ryan et al., 1985). Formalin produces a dose-related increase in pain behaviours in mice (Rosland et al., 1990) and rats (Coderre et al., 1993; Abbott et al., 1995). While it has been suggested that the use of low formalin concentrations may increase the sensitivity of this test (Tjølsen et al., 1992), the role of stimulus intensity in determining pharmacological outcome has received only limited attention to date (Hunskaar et al., 1985; Rosland et al., 1990).

In the present study, we have examined the effects of exogenously administered adenosine analogs (N^6-cyclohexyladenosine which is adenosine A_1 receptor selective, 2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS-21680) which is adenosine A_{2A} receptor selective, and 5′-*N*-ethylcarboxamidoadenosine (NECA) which is non-selective for adenosine A_1 and A_2 receptors) at different formalin concentrations. In addition, the effects of inhibitors of adenosine metabolism via adenosine kinase (by 5′-amino-5′-deoxyadenosine) or adenosine deaminase (by deoxycoformycin) were examined using different intensities of stimulation to determine if effects of endogenous adenosine are expressed only following mild intensity stimulation, as suggested by intensity-dependent hyperalgesia with methylxanthines.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (Charles River Laboratories) weighing 100–125 g were used. They were housed in groups of 2–3 in a room maintained at 21–23°C with free access to food and water on a 12/12 h light cycle. Each animal was tested only once.

2.2. Drug administration

Before the start of an experiment, the animal was habituated in the plexiglass observation chamber (30 × 30 × 30 cm) for 15 min. The agent tested was administered intrathecally by percutaneous lumbar puncture (Hylden and Wilcox, 1980) under halothane anaesthesia using a 50 μl microinjection syringe and a 30 gauge needle. A tail flick indicated penetration of the subarachnoid space. Following injection of 20 μl of drug solution, the animal was returned to the chamber for recovery. Animals recovered from anaesthesia within minutes. In antagonist experiments, caffeine was mixed with the agonist and 20 μl of the resulting solution was administered.

2.3. Formalin test

10 min after drug administration, 50 μl of formalin (2% or 5%) was injected subcutaneously into the dorsum of the left hind paw of the unanaesthetized rat using a 30 gauge needle. The animal was then returned to the chamber for observation. A mirror was placed behind the chamber to enable unhindered observation of the formalin-injected paw. Two forms of activity, flinching/lifting and licking/biting of the injected paw, were recorded as pain behaviours (Wheeler-Aceto and Cowan, 1991). The number of flinches/lifts and the

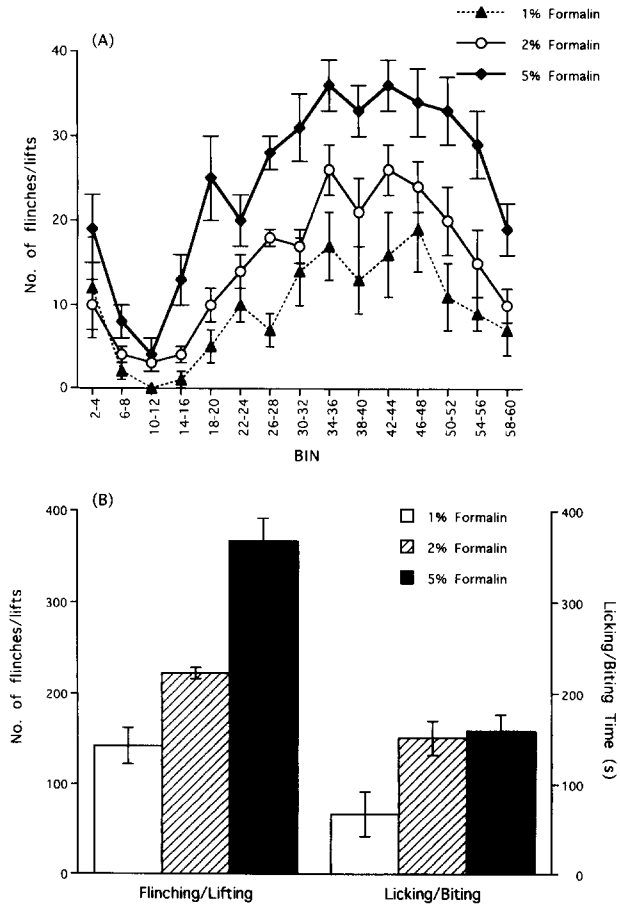

Fig. 1. (A) Time course for the flinching/lifting behaviour induced by different concentrations of formalin injected subcutaneously into the dorsal hind paw of the rat. Formalin was injected at 0 min and each bin represents a 2-min observation period. (B) Cumulative number of flinches/lifts and licking/biting time in both phases (0–60 min) with various formalin concentrations. For both panels, values are mean ± S.E.M. (n = 6–12).

amount of time spent licking/biting within 2-min periods were recorded over a total test duration of 60 min. Two animals were observed in alternate bins. The animal was killed at the end of the test.

2.4. Drugs

The following drugs were used in this study: N^6-cyclohexyladenosine (Research Biochemicals); 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS-21680 hydrochloride; Research Biochemicals); 5′-*N*-ethylcarboxamidoadenosine hydrate (NECA; Research Biochemicals); 5′-amino-5′-deoxyadenosine (*p*-toluenesulfonate salt) (Sigma Chemical Co.); deoxycoformycin (Parke-Davis Pharmaceutical Research Division, Warner-Lambert Co.); caffeine (Sigma Chemical Co.). All drugs were dissolved in normal saline except CGS-21680, which was dissolved in 5% dimethylsulfoxide (DMSO; Sigma Chemical Co.). Formalin solutions (2% or 5%) were prepared by diluting a 37% formaldehyde stock solution (Sigma Chemical Co.) with normal saline.

2.5. Statistical analysis

Statistical significance in multiple comparisons was established by analysis of variance (ANOVA) followed by the Least Significant Difference post-hoc analysis. Level of significance was set at 0.05.

3. Results

3.1. Formalin-induced behaviours

Subcutaneous injection of 1–5% formalin resulted in a characteristic biphasic response (Fig. 1A). Phase 1 (early phase) starts immediately after injection of formalin and lasts for 10 min, whereas the period from 10 to 60 min after formalin injection constitutes phase 2 (the late phase). Flinching was continuously expressed throughout the 60-min observation period, but licking/biting episodes were observed predominantly during phase 2. Licking/biting behaviour was maximally expressed at 2% formalin, while flinching behaviour was further enhanced at the 5% formalin concentration (Fig. 1B).

3.2. Effects of adenosine-receptor agonists on formalin-induced responses

N^6-Cyclohexyladenosine (adenosine A_1 receptor agonist), CGS-21680 (adenosine A_{2A} receptor agonist) and NECA (adenosine A_1 and A_2 receptor agonist) were tested at doses of 0.1–10 nmol. At 10 nmol, both N^6-cyclohexyladenosine and NECA produced marked motor impairment or flaccid paralysis of the hind limbs, and these animals were excluded from the analysis. Exploratory behaviour was observed throughout, and it was noted that animals given N^6-cyclohexyladenosine

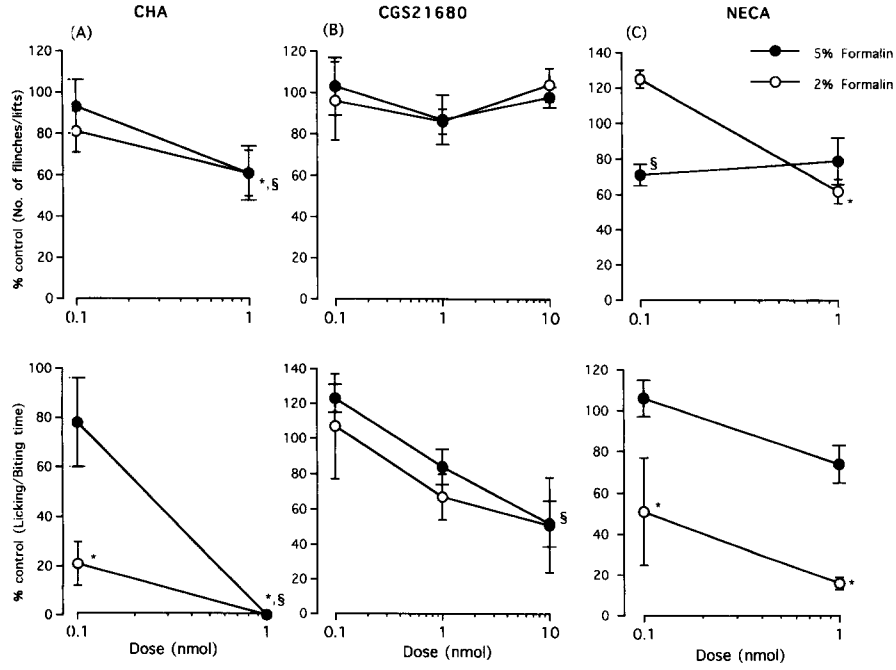

Fig. 2. Dose-related inhibition of the 2% formalin phase 2 (10–60 min) response for (A) N^6-cyclohexyladenosine (CHA), (B) CGS21680 and (C) NECA. Response expressed as percentage of the cumulative mean control value for phase 2. Statistical calculations based on raw data. * and § denote $P < 0.05$ compared to control in the 2% and 5% groups respectively ($n = 4$–6 per group).

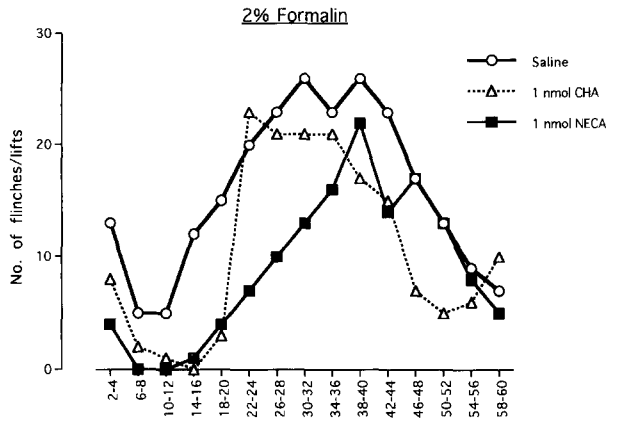

Fig. 3. Biphasic time course for flinching/lifting behaviour in response to 2% formalin following pre-treatment (10 min before formalin injection) with N^6-cyclohexyladenosine (CHA) and NECA. Values represent the mean for $n = 15$ (saline) or 4–6 (CHA, NECA) per group.

or NECA appeared less active than control animals. CGS-21680 did not produce any visible impairment of motor function or alteration in locomotor activity at any dose. None of the agents induced any significant change in phase 1 scores (data not shown), so only phase 2 scores are presented. N^6-cyclohexyladenosine (1 nmol) suppressed flinching/lifting behaviour in animals injected with both 2% and 5% formalin (Fig. 2A and Fig. 3). When licking/biting was measured, both 0.1 and 1 nmol doses reduced activity in the 2% group, whereas in the 5% group, only 1 nmol N^6-cyclohexyladenosine was effective. All doses of CGS-21680 failed to reduce the frequency of flinching/lifting in either the 2% or 5% formalin group, but a limited amount of suppression of licking/biting was observed with 10 nmol (Fig. 2B). NECA produced a modest reduction in flinching/lifting, but a much greater reduction in licking/biting time was observed when 2% formalin was used (Fig. 2C and Fig. 3).

3.3. Effects of caffeine on a directly acting adenosine agonist

In order to show that the behavioural effects produced by adenosine analogues were mediated through adenosine receptors, caffeine, a non-selective adenosine-receptor antagonist, was used to block the effects of NECA. Caffeine, at doses of 50 and 100 μg, administered intrathecally, did not produce any significant change in either flinching/lifting or licking/biting in 2% formalin groups (Fig. 4). When co-injected with NECA, both doses of caffeine completely blocked suppression of flinching/lifting by NECA, but only the higher dose blocked the effect of NECA on licking/biting (Fig. 4). Blockade was essentially complete as there was no difference between the saline and the caffeine 100 μg/NECA group.

3.4. Effects of 5'-amino-5'-deoxyadenosine and deoxycoformycin on formalin-induced responses

None of the animals injected with 5'-amino-5'-deoxyadenosine or deoxycoformycin exhibited signs of motor impairment or reduced locomotor activity. When 5% formalin was used, all doses of 5'-amino-5'-deoxyadenosine failed to reduce either the number of flinches/lifts or licking/biting time (Fig. 5A). However, using 2% formalin, 5'-amino-5'-deoxyadenosine suppressed both flinching/lifting and licking/biting (Fig. 5A and Fig. 6). On the other hand, deoxycoformycin failed to significantly alter both formalin-induced responses at all doses and formalin concentrations (Fig. 5B).

3.5. Effects of caffeine on 5'-amino-5'-deoxyadenosine

In order to determine whether 5'-amino-5'-deoxyadenosine produced its effects by increasing levels of

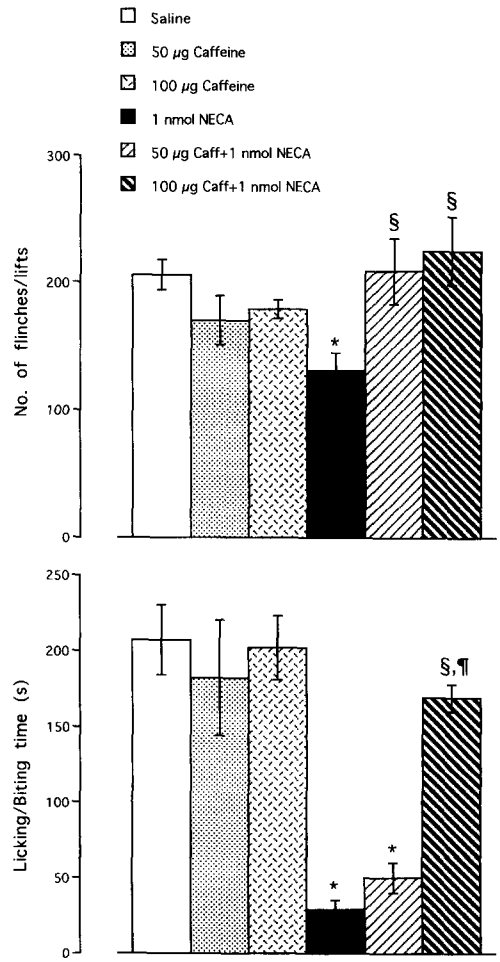

Fig. 4. Caffeine (Caff) blocks suppression of 2% formalin phase 2 responses by NECA. * $P < 0.05$ compared to control group; § $P < 0.05$ compared to 1 nmol NECA group; ¶ $P < 0.05$ compared to 50 μg caffeine + 1 nmol NECA group ($n = 4$–9).

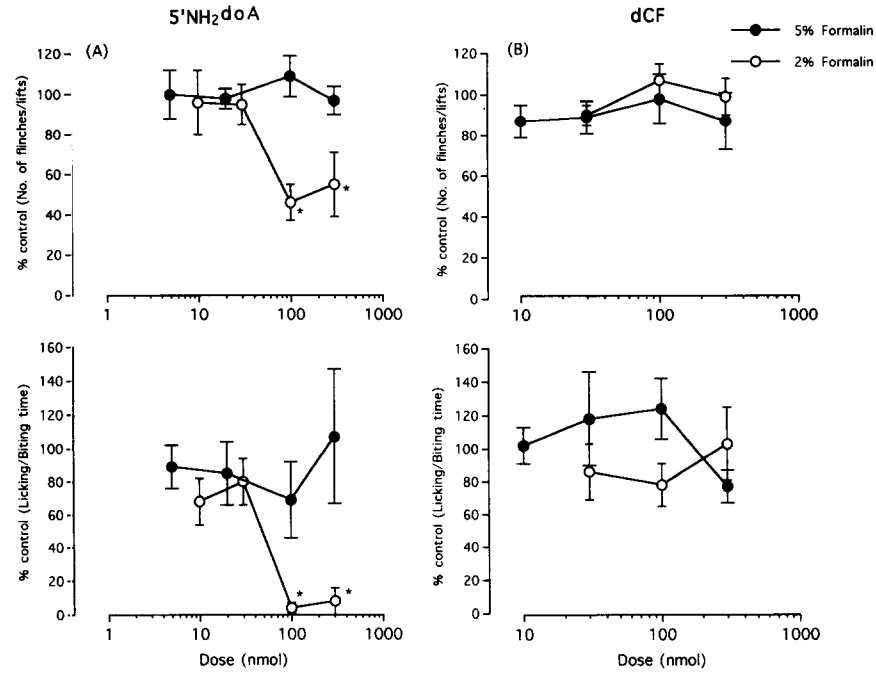

Fig. 5. Phase 2 formalin responses were inhibited by (A) 5'-amino-5'-deoxyadenosine (5'NH_2doA), an adenosine kinase inhibitor, but not (B) deoxycoformycin (dCF), an adenosine deaminase inhibitor in the 2% (but not 5%) formalin groups. * $P < 0.05$ compared to control values ($n = 4–9$).

endogenous adenosine which then act on cell surface adenosine receptors, the effects of caffeine on the action of 5'-amino-5'-deoxyadenosine were assessed. Co-administration of either 50 or 100 μg caffeine with 5'-amino-5'-deoxyadenosine reversed suppression of the flinching/lifting response by the adenosine kinase inhibitor, but both doses of caffeine failed to block the action of 5'-amino-5'-deoxyadenosine on the licking/biting response (Fig. 7).

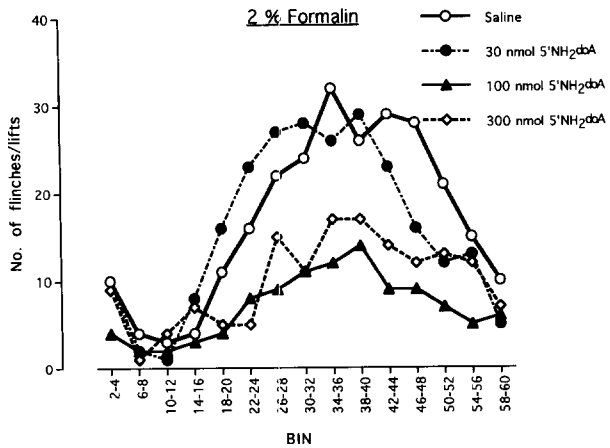

Fig. 6. Biphasic time course for flinching/lifting behaviour in response to 2% formalin following pre-treatment (10 min before formalin injection) with 5'-amino-5'-deoxyadenosine (5'NH_2doA). Values represent the mean for $n = 15$ (saline) or 4–6 per group.

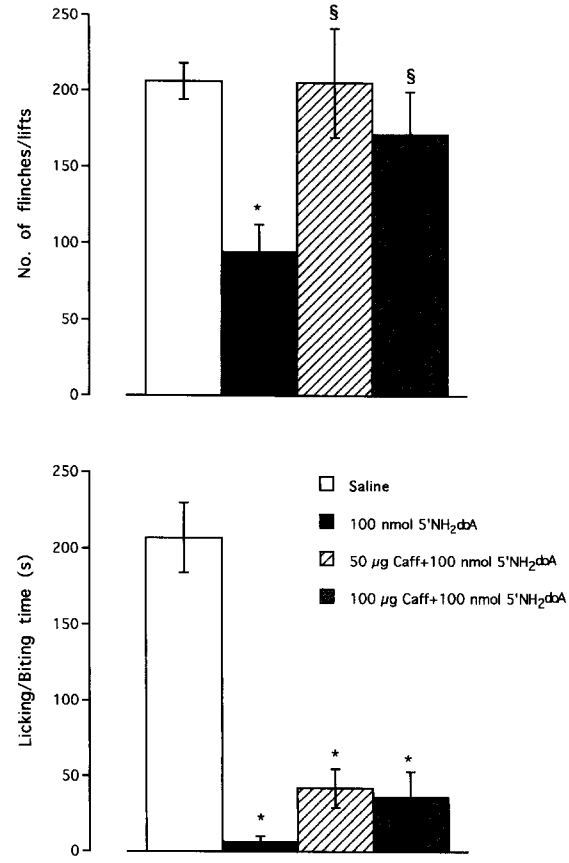

Fig. 7. Reversal of 5'-amino-5'-deoxyadenosine (5'NH_2doA) induced suppression of 2% formalin phase 2 responses by 50 and 100 μg caffeine (Caff). * $P < 0.05$ compared to saline controls; $ $P < 0.05$ compared to 5'NH_2doA group ($n = 4–9$).

4. Discussion

The present study demonstrated that N^6-cyclohexyladenosine (adenosine A_1 receptor selective), NECA (A_1/A_2) and 5'-amino-5'-deoxyadenosine (adenosine kinase inhibitor) produced antinociception in the rat formalin model, and that such antinociception was mediated via activation of spinal adenosine receptors. Antinociception was dependent upon stimulus intensity, an effect seen most clearly in the case of 5'-amino-5'-deoxyadenosine. The relative inability of CGS-21680 (adenosine A_{2A} receptor selective) to produce antinociception suggests that spinal adenosine A_1 receptors are more important than adenosine A_{2A} receptors in regulating antinociception. The inability of the adenosine deaminase inhibitor deoxycoformycin to produce antinociception suggests that adenosine kinase is a more important regulator of endogenous adenosine levels in the spinal cord than adenosine deaminase.

The spinally mediated analgesic activity of adenosine analogues in thermal threshold tests was initially attributed to activation of both adenosine A_1 and A_2 receptors (Post, 1984; Sawynok et al., 1986; Holmgren et al., 1986; DeLander and Hopkins, 1987). The distribution of both adenosine A_1 and A_2 receptor populations in the substantia gelatinosa of the dorsal horn of the spinal cord (Geiger et al., 1984; Choca et al., 1987) is consistent with this proposed involvement. Here we demonstrated that exogenous administration of adenosine agonists with activity at adenosine A_1 receptors (N^6-cyclohexyladenosine and NECA) produced antinociception in the rat formalin test as reported previously with another such agonist, L-phenylisopropyladenosine (Malmberg and Yaksh, 1993). The relative lack of effect of CGS-21680 suggests that adenosine A_{2A} receptors do not mediate spinal antinociception. Other studies have similarly demonstrated that selective adenosine A_2 agonists do not produce antinociception (DeLander and Wahl, 1988; Karlsten et al., 1991), and emphasized that antinociception correlates with affinity of ligands at adenosine A_1 receptors (Karlsten et al., 1991). The spinal mechanism by which adenosine A_1 receptor activation produces antinociception is not clearly established. A postsynaptic inhibition on transmission neurons has been implicated (DeLander and Wahl, 1988; Salter et al., 1993), and this is consistent with a predominant postsynaptic localization of receptors (Choca et al., 1987, 1988). There is some evidence for a presynaptic action to inhibit neuropeptide release (Santicioli et al., 1992) but this has not been consistently observed (Vasko and Ono, 1990). Additional effects of adenosine on interneurons in the dorsal horn have also been suggested (Sah, 1990; Li and Perl, 1994). Activation of adenosine A_1 receptors engages G_i, inhibits adenylate cyclase, and increases K^+ and decreases Ca^{2+} conductance (Fredholm et al., 1994). Pertussis toxin sensitive G-proteins, inhibition of adenylate cyclase (Sawynok and Reid, 1988) and activation of K^+ channels (Salter et al., 1992) have variously been implicated in antinociception by adenosine within the spinal cord. Activation of adenosine A_2 receptors produces stimulation of adenylate cyclase (Fredholm et al., 1994) and excitatory effects in neural tissue (O'Regan et al., 1992, Sebastião and Robeiro, 1992). It is not clear how such stimulatory effects could mediate spinal analgesia. The possibility of stimulation of an inhibitory interneuron in the substantia gelatinosa exists, but there is no evidence to support this notion.

The present study has demonstrated that inhibition of adenosine kinase, but not adenosine deaminase, produces antinociception in the formalin test. This result is similar to that observed using a thermal threshold test (Keil and DeLander, 1992). It appears that adenosine kinase is more important than adenosine deaminase in regulating endogenous adenosine levels in the dorsal spinal cord, as noted for brain (Murray et al., 1993; Zhang et al., 1993). Adenosine deaminase has been localized to the substantia gelatinosa of the dorsal spinal cord (Nagy and Daddona, 1985), but no such localization data are available for adenosine kinase. The effects of the adenosine kinase inhibitor were highly dependent on stimulus intensity (see below), and at 2% formalin, the magnitude of reduction in behaviours was comparable to or greater than that achieved by the exogenously added agonists. The effects of 5'-amino-5'-deoxyadenosine were fully or partly reversed by caffeine, implying the involvement of adenosine receptors. The reduction in licking/biting behaviour with NECA was less sensitive to caffeine reversal, but the limited degree of reversal of inhibition of licking/biting behaviour by 5'-amino-5'-deoxyadenosine with caffeine was surprising and raises the possibility of actions of 5'-amino-5'-deoxyadenosine independent of adenosine kinase inhibition. Interestingly, antinociception by 5'-amino-5'-deoxyadenosine was not accompanied by impairment of motor function at any dose. This is in contrast to exogenously administered adenosine analogs in rats which consistently produce motor effects as doses are increased to 3–10 nmol (Post, 1984; Sawynok et al., 1986; Malmberg and Yaksh, 1993). The profile of action produced by indirectly acting agents suggests that a selective action of adenosine within the dorsal horn to regulate sensory transmission can be achieved without accompanying effects in the ventral horn on motor function.

An interesting finding of our study is the influence of stimulus intensity on drug effects. Formalin-induced responses in rodents are related to the concentration of formalin injected (Rosland et al., 1990; Coderre et al., 1993; Abbott et al., 1995). For exogenously admin-

istered adenosine agonists, there is little dependence on formalin concentration for flinching behaviour (cf. Malmberg and Yaksh, 1993), but for licking/biting behaviour, there is a clear dependence on stimulus intensity. With 5'-amino-5'-deoxyadenosine, both pain-related responses produced by 2% formalin were reduced, but this was not seen when 5% formalin is employed. Although 5% formalin has been used most frequently in rats as the pain stimulus (Tjølsen et al., 1992), little data are available to confirm its advantage over lower concentrations of formalin. The issue of stimulus intensity is perhaps less important when powerful analgesics such as the opioids are tested (Rosland et al., 1990). However, the detection of the effects of weaker analgesics or certain classes of analgesics may be facilitated when lower formalin concentrations are used, as the sensitivity of the test is increased (Shibata et al., 1989; Tjølsen et al., 1992). For example, indomethacin-induced antinociception in mice was demonstrated using 1% formalin, but not when 5% formalin was used (Rosland et al., 1990). Since 5'-amino-5'-deoxyadenosine produces an indirect action and may be considered a weak analgesic as it is dependent on an endogenously driven tone, the use of a lower formalin concentration in this case increases the sensitivity of the test. It appears that endogenously released adenosine in the spinal cord may play a modulatory role in nociceptive processing only at certain intensities of stimulation.

In this study, we measured both flinching/lifting and licking/biting behaviours. Pain is a complex perception with multidimensional properties, and in animals can be regarded as a composite of several nociceptive behaviours (Tjølsen et al., 1992). Flinching/lifting and licking/biting behaviours have been established to be consistently correlated with pain (Wheeler-Aceto and Cowan, 1991; Abbott et al., 1995). Other behavioural patterns such as favouring, grooming and rearing may also be related to nociception, but their relationship with noxious stimulation is less distinct. Our results indicate that these two behavioural parameters in rat can be differentially modulated at the spinal level. Dissociations in the ability of spinally administered agents to differentially affect these behaviours have been noted previously (Wheeler-Aceto and Cowan, 1993). Both licking and late phase flinching are supraspinally integrated responses (Wheeler-Aceto and Cowan, 1991); however, examination of the time courses of flinching/lifting and licking/biting reveals two clearly different temporal patterns. Except for the short 'interphase' of inactivity, flinching/lifting is a continuous and rhythmic behaviour, whereas licking/biting is sporadic and each episode lasts only for a brief period. This indicates that flinching/lifting may be the result of a tonic, sub-acute pain sensation or discomfort, while licking/biting may represent episodic attacks of phasic pain. Whether the differences in action of adenosine analogs on the two responses (greater degree of inhibition of licking/biting for exogenously administered agents) reflects upon potential different actions of adenosine in modulating sensory transmission in the spinal cord (via actions at different sites, see above) remains to be determined.

Acknowledgements

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