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The roles of spinal adenosine receptors in the control of acute and more persistent nociceptive responses of dorsal horn neurones in the anaesthetized rat

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1 We describe here the effects of intrathecal selective adenosine receptor agonists on acute and more persistent evoked responses of dorsal horn nociceptive neurones recorded in intact rats anaesthetized with halothane.

2 The effects of the A_1 receptor agaonist, N^6-cyclopentyladenosine and the non-selective agonist 2-chloroadenosine as well as the A_{2a} receptor agonist, 2-*p*-(2-carboxyethyl) phenethylamino-5'-*N*-ethylcarboxamidoadenosine hydrochloride were gauged on the C-, Aδ-, Aβ-fibre, post-discharge and wind-up responses produced by peripheral tanscutaneous stimulation. The antagonists, theophylline and 8(*p*-sulphophenyl) theophylline were also tested alone and to reverse the agonist effects.

3 Subcutaneous formalin (5%) was used to produce a more prolonged nociceptive response initiated by peripheral inflammation.

4 Both N^6-cyclopentyladenosine and 2-chloroadenosine produced inhibitions of the C-fibre evoked responses, wind-up and post-discharge of the neurones with no significant effects on the Aβ responses. By contrast, the Aδ evoked responses were facilitated over the same time course and dose-range as the inhibitions. N^6-cyclopentyladenosine was more potent and effective than 2-chloroadenosine. In marked contrast to these agonists, the A_{2a} agonist produced only weak non-specific inhibitions. Theophylline and 8(*p*-sulphophenyl) theophylline alone had no effect on the acute responses but prevented or reversed inhibitory effects of N^6-cyclopentyladenosine.

5 The formalin response was markedly inhibited by spinal N^6-cyclopentyladenosine with both the acute first phase and more prolonged second phase being dose-dependently inhibited. N^6-cyclopentyladenosine was considerably more potent on the formalin response than on the other neuronal measures.

6 The results suggest a role of adenosine A_1 receptors in the modulation of both acute and inflammatory nociception in the spinal cord.

Keywords: Antinociception; adenosine A_1-receptors; N^6-cyclopentyladenosine; spinal cord; theophylline; formalin response; adenosine A_{2a}-receptors

Introduction

The purine, adenosine, may have important roles in the control of nociception. Since the original observations (Paalzow & Paalzow, 1973) where methylxanthine adenosine antagonists decreased nociceptive thresholds in rats, increasing evidence has indicated that spinal adenosine plays a role in the modulation of nociceptive processing in the spinal cord (Sawynok & Sweeney, 1989). Previous behavioural studies have shown that spinal adenosine agonists are antinociceptive (Sosnowski & Yaksh, 1989; Karlsten *et al.*, 1990; Malmberg & Yaksh, 1993). There is however, little functional *in vivo* electrophysiological data on the roles of adenosine in antinociception (Salter & Henry, 1985; 1987) and none regarding the control of different responses of spinal nociceptive neurones to acute and more persistent stimuli.

Adenosine-like immunoreactivity is exhibited by terminals in the substantia gelatinosa of the dorsal horn of the spinal cord (Braas *et al.*, 1986) where high levels of adenosine receptors are found (Goodman & Snyder, 1982) including the inhibitory A_1 receptor (Geiger *et al.*, 1984). Adenosine has been suggested to have both pre- and postsynaptic effects within the dorsal horn (see Sawynok & Sweeney, 1989; Li & Perl, 1994) indicating possible multiple sites of action of adenosine in the control of sensory events related to pain.

There is evidence that adenosine may be released by, and can act to control N-methyl-D-aspartate (NMDA)-mediated events in the cortex (Hoehn & White, 1990; Craig & White, 1992, 1993) and hippocampus (Manzoni *et al.*, 1994). It is known that activation of the NMDA receptor mediates wind-up and the post discharge of nociceptive neurones in the spinal cord, events that enhance and prolong more persistent pain states (Davies & Lodge, 1987; Dickenson & Sullivan, 1987; Dubner & Ruda, 1992; McMahon *et al.*, 1993; Dickenson, 1994a; Price *et al.*, 1994). We hypothesize that adenosine may act to control NMDA events in nociception at the spinal level.

Here we examine the effects of intrathecal A_1 adenosine receptor agaonists, N^6-cyclopentyladenosine and the less selective agonist, 2-chloroadenosine as well as the A_{2a} receptor agonist, 2-*p*-(2-carboxyethyl) phenethylamino-5'-*N*-ethylcarboxamindoadenosine hydrochloride (CGS-21680). In addition, the antagonists, theophylline and 8(*p*-sulphophenyl) theophylline were tested alone and against the effects of agonists on the electrically evoked A- and C-fibre activity of deep dorsal horn nociceptive neurones. We were particularly interested in the post-discharge and wind-up responses of the neurones since these are known to be NMDA-mediated (see Dickenson, 1994a). In addition to these acute measures of nociception the neuronal activity as a result of peripheral injection of formalin was observed, producing a more persistent inflammatory response which is also spinally mediated by NMDA receptor activation (Haley *et al.*, 1990).

Methods

Animal preparation

The methods used are essentially those previously described (Dickenson & Sullivan, 1986). Male Sprague-Dawley rats

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(200–250 g) were anaesthetized with 3% halothane in a 66% N_2O and 33% O_2 mixture. Once anaesthesia was induced, it was administered via a nose cone with 2–2.5% halothane. The trachea was opened, a cannula placed into the trachea and securely fastened with silk thread. The anaesthetic was then administered directly into the cannula in order to maintain stable conditions during the experiment.

The animal was held in ear bars in a stereotaxic frame, and a laminectomy performed over lumbar segments L1-L3 to expose the spinal cord. Once the surgery was finished the animal was maintained on 1.5% halothane for the duration of the experiment. Body temperature of the animal was controlled via a heating blanket placed under the animal with a feedback system using a rectal probe.

Experimental procedure

Doral horn cells with receptive fields on the ipsilateral hindpaw, mainly the toes, were isolated using parylene coated tungsten electrodes which were inserted into the most superficial spinal cord and then moved in a vertical plane by means of a micordrive. Cell depths were measured to within 10 μm. All the cells responded to innocuous (touch and prod) and noxious (pinch) peripheral stimulation. The receptive field was stimulated with electrical stimulation to activate the neurones. A trial consisted of a train of 16 impulses given every 10 min at three times the C-fibre threshold and again at three times the Aβ-fibre threshold for the agonist studies on the electrically evoked responses. Stimulation was at 0.5 Hz with a 2 ms wide pulse.

Post stimulus histograms of the response in each trial were produced, from which the responses due to the different fibres types, were separated on the basis of threshold and latency so that the Aβ, Aδ-, C-fibre and post-discharge could be quantified (see Figure 1). The responses due to Aβ-stimulation were counted over a latency range of 0–20 ms, Aδ- from 20–90 ms and the C-fibre band from between 90–300 ms. As the train of 16 stimuli, at C-fibre strength was given, making up the trial, wind-up was observed in the large majority of neurones. The evoked responses to the first few stimuli were constant and no activity after 300 ms was observed. Wind-up, and NMDA-induced sudden increased excitability of the neurone despite the constant peripheral stimulus (Davies & Lodge, 1987; Dickenson & Sullivan, 1987) then occurred and resulted in enhanced evoked activity and the appearance of post-discharge activity following the main C-fibre response (see Figure 1 and controls in Figure 4). This post-discharge was counted over the latency period of 300–800 ms and this was used as an indirect measure of wind-up. Additionally wind-up was measured directly in the following way: the first response evoked by C-fibres, used as an indication of C-fibre input onto the neurone, was multiplied by 16 and subtracted from the total response to the 16 stimuli in the C-fibre and post-discharge latency band. This gives a value of the excess action potentials, elicited by wind-up, amplifying the initial response to the constant stimulation.

The drugs were added directly onto the exposed spinal cord in a volume of 50 μl immediately after stable control neuronal responses had been achieved (less than 10% variation). We have previously established that the saline vehicle has no effect on any of the neuronal measures over the timecourse of these studies. To reduce the number of animals used we did not include further saline controls in the present study. In addition, the lowest doses used were generally without effect, arguing against non-specific effects. Tests were done at 10 min intervals, for up to 40 min. The next dose was then administered and up to three doses tested cumulatively on one cell. Doses were incremented by a factor of ten. All results were then expressed as a percentage of the initial controls.

Formalin-induced activity

Electrical stimulation was also used to gauge the characteristics of the neurones prior to their inclusion in the formalin studies if a clear C-fibre evoked response was observed. Subsequent injection of 5% formalin (volume 50 μl) by the subcutaneous route into the peripheral receptive field, (always a toe) of neurones with C-fibre responses, resulted in instantaneous firing of the deep dorsal horn cells, which beforehand had little or no spontaneous activity. The action potentials elicited in the first 10 min were counted as a measure of the first phase, which was followed by a silent or much quieter periods lasting a few minutes. The second phase of firing commenced after about 20 min and often continued beyond the 60 min recording period. The second phase was quantified as action potentials occurring between 10 and 60 min after the initial injection of formalin. Drugs were applied 20 min before the injection of formalin, and only one dose was tested per formalin response.

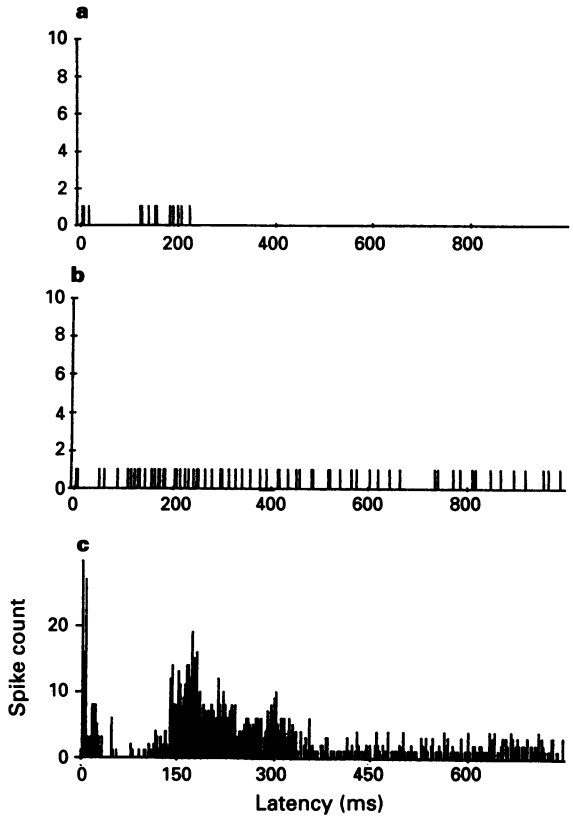

Figure 1 An example of post-stimulus histograms showing the cumulative response of a neurone to the train of 16 stimuli, given at three times C-fibre threshold with a 2 ms wide pulse; (a) The responses evoked by the first stimulation; (b) the post-stimulus histogram to the 16th stimulation, showing wind-up; (c) the post-stimulus histogram to all 16 stimuli. The responses due to activation of different fibre types are separated on basis of latency; the Aβ-fibre evoked responses occur between 0–20 ms, Aδ-fibres between 20–90 ms, C-fibres between 90–300 ms and the post-discharge between 300–800 ms.

Data analysis

Student's *t*-test and the Mann-Whitney test were used to analyse results. Student's *t*-test was a paired, two tailed test, and with both tests the significance level was set at a P value of 0.05.

Drugs

All drugs were given by intrathecal route. N^6-cyclopentyladenosine (N^6-CPA) 2-chloroadenosine (2-Cl-Ado) and theophylline were all obtained from Sigma, Dorset, England. 2-*p*-(2-Carboxyethyl) phenethylamino-5'-*N*-ethylcarboxamidoadenosine hydrochloride (CGS-21680) and 8(*p*-sulphophenyl) theophylline (8-*p*SPT) were obtained from Research Bio-

chemicals International, Natick, MA 01760, U.S.A. All drugs were diluted in 0.9% saline. Formalin was diluted with saline from a stock of 38.5% formalin, to produce a 5% formalin solution. Formalin was obtained from BDH chemicals, Leicestershire.

Results

N^6-cyclopentyladenosine

In this series of experiments the effects of intrathecal N^6-CPA were investigated on the responses of a total population of 18 doral horn neurones, recorded at a mean depth of 740 μm $\pm$ 42 from the surface of the spinal cord. Intrathecally administered N^6-CPA at doses of 0.05, 0.5, 5, 50 and 250 μg was evaluated on the population of cells. In the calculations of the overall results, 2 cells were omitted since they were spontaneously active throughout the experiment.

N^6-CPA dose-dependently inhibited the C-fibre evoked response, post-discharge and wind-up of the neurones (see Figure 2a and b and Figure 4 for individual examples). The lowest doses tested (0.05 μg and 0.5 μg) had no effect on any of these responses ($P > 0.05$). As the doses were increased to 5 μg the responses evoked by the C-fibres were significantly inhibited by $31 \pm 10\%$ ($P < 0.01$) and the post-discharges also tended to be reduced by $30 \pm 19\%$. With 50 μg both responses were markedly inhibited ($P < 0.005$) with the C-fibres being reduced to $55 \pm 7\%$ of controls and the post-discharges to $36 \pm 12\%$. Wind-up was also dose-dependently reduced by the agonist. Responses elicited by Aβ-fibre stimulation were not significantly changed with any of the five doses administered ($P > 0.05$) so that even with the highest dose of 250 μg only a $23 \pm 24\%$ inhibition was observed. Interestingly, as the C-fibre evoked response, post-discharge and wind-up of the neurones were inhibited by increasing doses of N^6-CPA, the Aδ-fibre evoked responses were facilitated by the same doses. The low doses of 0.05 and 0.5 μg had no effect, but increasing the dose to 5 μg and above produced clear facilitations ($P < 0.05$) so that the Aδ-fibre response after 250 μg N^6-CPA was increased by $89 \pm 29\%$.

The effect of each dose was monitored for a 40 min period. The inhibition of the C-fibre and post-discharge responses with 5 and 50 μg was maximal at 20 min. The highest dose (250 μg) produced maximal inhibition at 40 min (see Figures 3a and b). The inhibitions produced by the effective doses of N^6-CPA on wind-up reached maximum between 10 and 40 min after drug administration. The Aβ-evoked response was not significantly altered even after the highest dose of 250 μg at any time point. The facilitatory effect on the Aδ-fibre evoked response with all doses was apparent by 10 min after treatment and then only showed small further increases (see Figure 3a).

Wind-up was inhibited by 50 μg N^6-CPA and examples are shown in Figure 4. In general, although there was some variability in the degree of inhibition, this dose reduced the wind-up of all of the neurones (Figure 2b). As the dose was increased to 250 μg wind-up was almost abolished but now the initial input was also dramatically reduced.

2-Chloroadenosine

In contrast to the response of N^6-CPA, the effects of 2-Cl-Ado, on a population of 6 cells, did not show a clear dose-dependency. The lowest dose tested (50 μg) produced significant inhibition of C-fibre evoked activity, post-discharge and wind-up (34 ± 10, 50 ± 11 and $33 \pm 9\%$ respectively) ($P < 0.05$) (see Figure 5). Subsequent doses (250 and 500 μg) failed to increase the inhibitions further. Aβ-fibre evoked responses showed no changes from control values, so that for example, the inhibition with the highest dose of 500 μg was only $20 \pm 24\%$. The Aδ-fibres were again facilitated so that a $61 \pm 18\%$ increase compared to controls was seen with 250 μg ($P = 0.01$). This facilitation mirrored the inhibitory effects of 2-Cl-Ado in time-course and amplitude, as seen with N^6-CPA. N^6-CPA, being more effective than 2-Cl-Ado was therefore used in the subsequent formalin studies.

CGS-21680 hyrochloride

The effects of CGS-21680 were studied on the electrically evoked responses to C-, Aδ- and Aβ-fibre stimulation of 8 dorsal horn neurones located 842 ± 60 μm deep in the spinal cord. CGS-21680 was applied intrathecally at doses of 0.75, 7.5, 25 and 75 μg and the effects followed for a 40 min period. Higher doses were not tested due to insolubility. Maximal effects were seen after 20 min but CGS-21680 did not distinguish between A- and C-fibre evoked responses. This inhibition of C-fibre and the Aβ-fibre evoked activity of deep dorsal horn neurones was only significant with the top dose of 75 μg giving an inhibition of $25 \pm 6\%$ and $41 \pm 5\%$ respectively ($P < 0.005$). The effects on post-discharge were not significant with any of the doses. The Aδ-fibre evoked responses were also inhibited by $27 \pm 4\%$ at 7.5 μg but increasing the doses failed to give rise

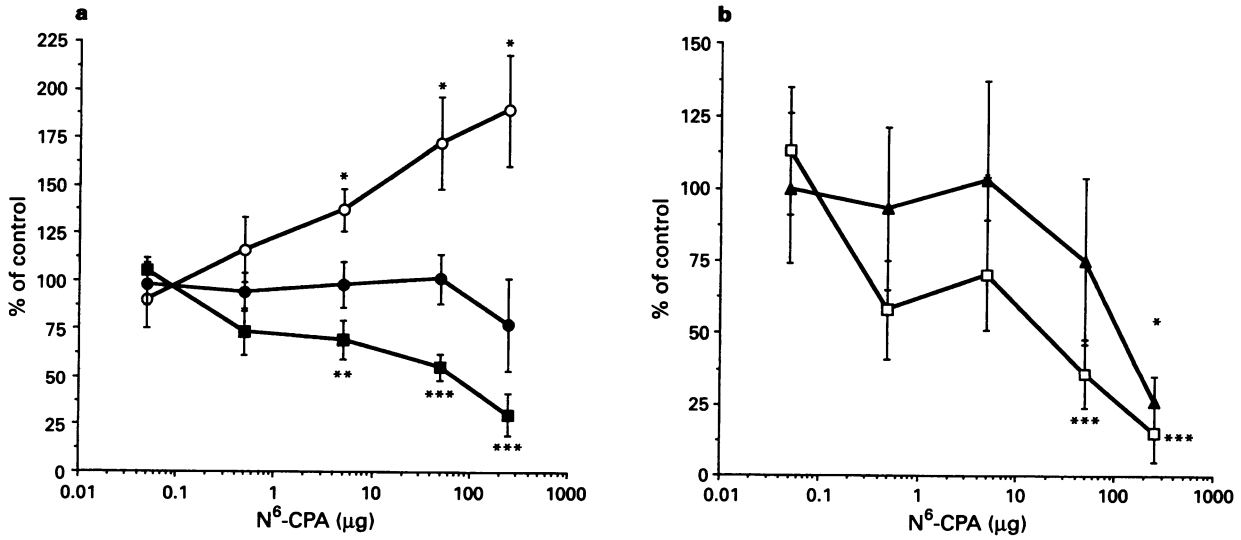

Figure 2 Dose-response relations for N^6-CPA. (a) Effects of 0.05, 0.5, 5, 50 and 250 μg N^6-CPA applied intrathecally on the response of dorsal horn neurones to electrically evoked C-fibre (■), Aδ-fibre (○) and Aβ-fibre (●) evoked activity. (b) Effects on the post-discharge (□) and wind-up (▲) of the same population of neurones. $*P = 0.05$; $**P < 0.01$; $***P < 0.005$.

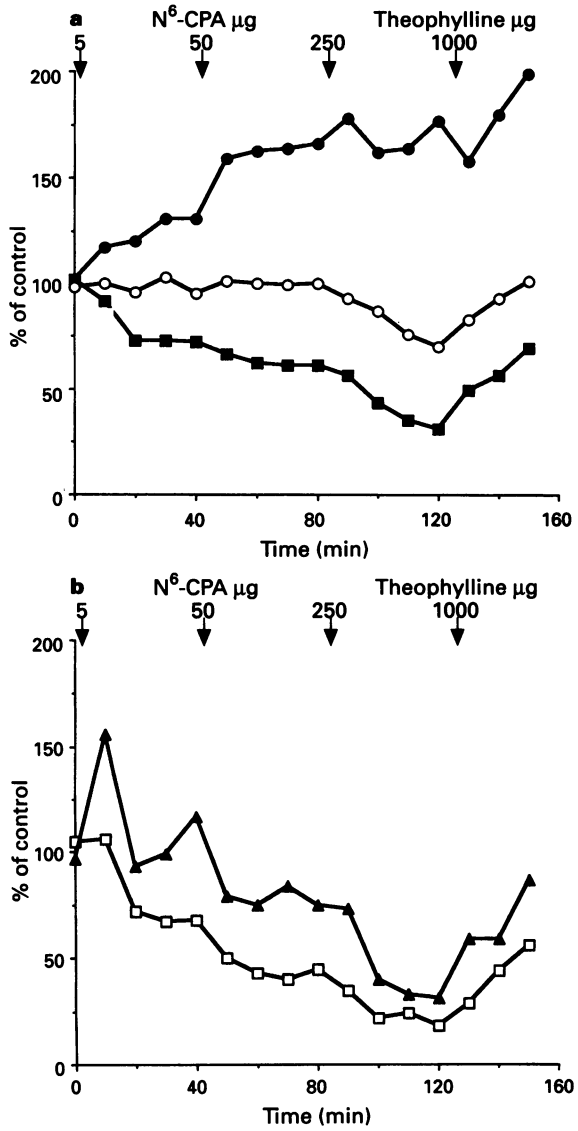

Figure 3 Time course and reversal by theophylline of the effects of N^6-CPA. Effects of 5, 50 and 250 μg of N^6-CPA applied intrathecally (as indicated by arrows) on the respones of dorsal horn neurones to electrically evoked (a) C-fibre (■), Aδ-fibre (●) and Aβ-fibre (○)-evoked activity. (b) post-discharge (□) and wind-up (▲). The reversal of these inhibitions by 1000 μg theophylline against time is also shown; however, note the facilitation of the Aδ-fibre evoked response was not reversed.

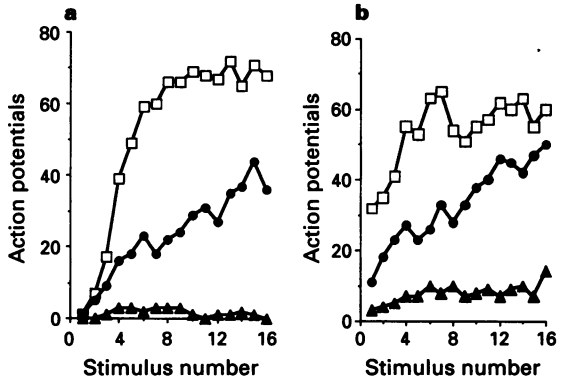

Figure 4 Wind-up of cells before and after the intrathecal administration of N^6-CPA. Two individual examples of evoked responses are plotted as action potentials against the stimulus number. The control response before drug application (□) was compared to 50 μg N^6-CPA (●) which reduced the wind-up of the neurones (the increase in evoked C-fibre responses to the constant stimulus). Whereas 250 μg (▲) abolished wind-up and as can clearly be seen for the neurone in (b), markedly reduced the initial responses of the neurones.

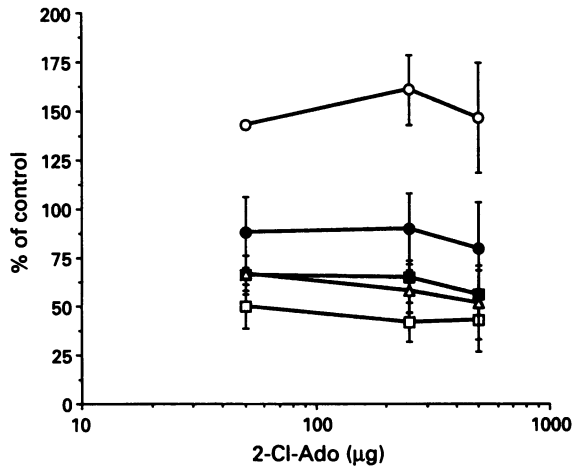

Figure 5 Dose-response relations for 2-Cl-Ado. Effects of intrathecally applied 2-Cl-Ado (50, 250 and 500 μg) on the responses of deep dorsal horn neurones classified as C-fibres (■), Aδ-fibres (○), Aβ-fibres (●), wind-up (△) and post-discharge (□) responses. $P=0.05$ for the effect of 50 μg 2-Cl-Ado on wind-up, post-discharge and for all doses on the C-fibre responses. The Aδ-fibre-evoked responses were facilitated, all doses being significant, $P=0.01$.

to greater inhibitions of responses (see Figure 6). These weak and non-selective inhibitions of both nociceptive and non-nociceptive responses contrast with the effects of N^6-CPA.

Theophylline

Intrathecal theophylline, at a dose of 1000 μg reversed the effects of both 250 μg N^6-CPA and 500 μg 2-Cl-Ado. It was also tested alone over a dose-range of 500–2000 μg on a population of 7 cells, previously untreated with any other compound. Theophylline tended to cause facilitations of the post-discharge, C-fibre evoked activity and wind-up but since the degree of increased activity was quite variable, the facilitations were not significant. Aβ and the Aδ-fibre evoked responses were also unchanged.

Theopylline (1000 μg) reversed the inhibitory effects of 250 μg N^6-CPA. This was maximal after 30 min for all responses of the 8 neurones studied. The inhibition of C-fibres, post-discharge and wind-up with 250 μg N^6-CPA was 70±11, 85±10, and 74±9% respectively and these inhibitions were reduced to 28±14, 40±17, and 5±29% respectively, after treatment with 1000 μg theophylline. In marked contrast, the facilitations of the Aδ-fibre evoked responses by 250 μg N^6-CPA were not reversed by theophylline and even tended to be enhanced. Thus, after treatment with 1000 μg theophylline the response was still facilitated by 99±33% compared to 89±29% with the agonist alone.

8(p-Sulphophenyl) theophylline

Intrathecal 8-pSPT was applied at doses of 1, 10, 100 and 400 μg on a population of 10 deep dorsal horn neurones (average depth 745±60 μm). 8-pSPT alone failed to have any significant effect on the electrically evoked C-, Aβ-, Aδ-fibre evoked responses, wind-up or post discharge of the neurones. However, when 400 μg 8-pSPT was given 40 min before 250 μg N^6-CPA, there was a significant reduction in the inhibitory effects of N^6-CPA on the C-fibre responses (70±11 control inhibition, 33±7% after the antagonist) and wind-up (71±9, 40±12% respectively) ($P<0.05$ for both). The weak

inhibitory effects of the agonist on the Aβ- responses were not influenced but importantly, the facilitations of the Aδ-fibre responses were unchanged by 8-pSPT (see Figure 7).

Formalin

An injection of 5% formalin (volume 50 μl) was made into the peripheral receptive field of 41 neurones. Twenty one cells were observed without any prior drug treatment, and used as controls. The first phase and second phase responses were 5248±1170 and 14530±3014 action potentials respectively.

Four doses of N^6-CPA (0.01, 0.1, 1, and 5 μg) were given intrathecally 20 min before the formalin injection. Five cells were observed for each dose (see Figures 8 and 9). The lowest dose (0.01 μg) had no effect on the first phase and only slightly inhibited the second phase (60±34% of control) but this was not significant. As the dose was increased the first phase of the formalin response was inhibited, so for example a reduction to 35±13% of control values ($P<0.05$) was seen with 0.1 μg N^6-CPA and 17±6% at 1 μg ($P<0.01$). Inhibition of the second phase was only significant at doses of 1 μg and above ($P<0.005$). At 5 μg the first and second phases were virtually abolished with responses of only 5±2 and 0.4±0.19% of control values being observed ($P<0.005$). The duration of the effects of the agonist increased with dose so that the partial recovery of the second phase of the formalin response seen after lower doses became a complete inhibition of the response after 5 μg N^6-CPA (Figure 9).

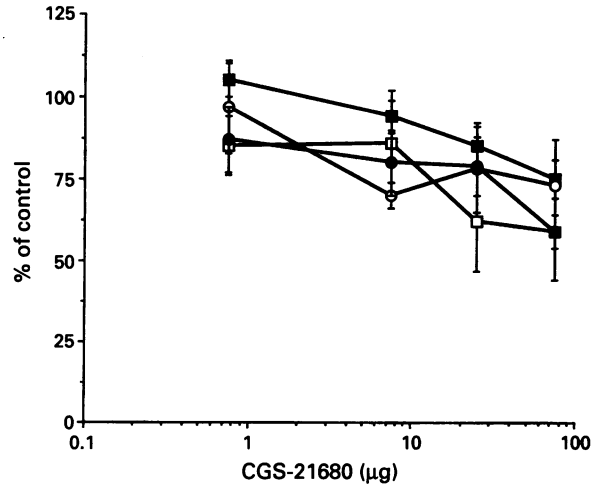

Figure 6 Dose-response relationship of the A_{2a}-agonist, CGS-21680, applied intrathecally (0.75, 7.5, 25, 75 μg). The response of the deep dorsal horn neurones ($n=8$) to C-fibre (■), Aδ-fibre (○), and Aβ-fibre (●) evoked responses, as well as post-discharge (□). Inhibition of the C- and Aβ-fibre evoked response was significant at 75 μg only. Inhibition of the Aδ-fibre response was significant at 7.5 μg.

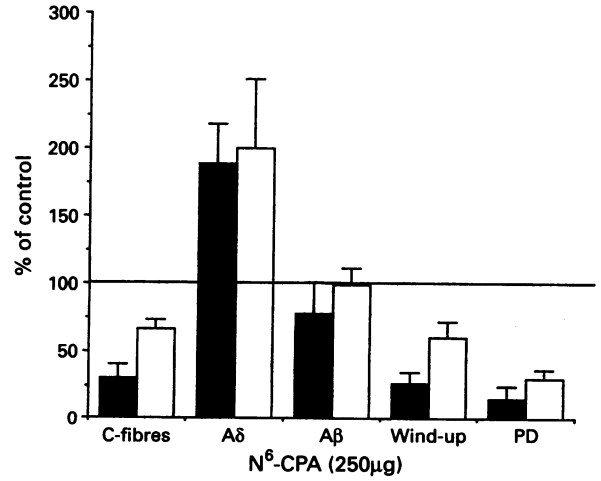

Figure 7 8-pSPT reverses some of the effects of N^6-CPA. The effect of N^6-CPA (250 μg) given alone (■), on the C-fibre, Aδ-fibre, Aβ-fibre, wind-up and post-discharge (PD) responses. Pretreatment of 8(*p*-sulphophenyl) theophylline (400 μg) was given 40 min prior to 250 μg N^6-CPA (□). The effects of the agonist on C-fibre-evoked responses and the wind-up were significantly attenuated by the pretreatment with 8-pSPT.

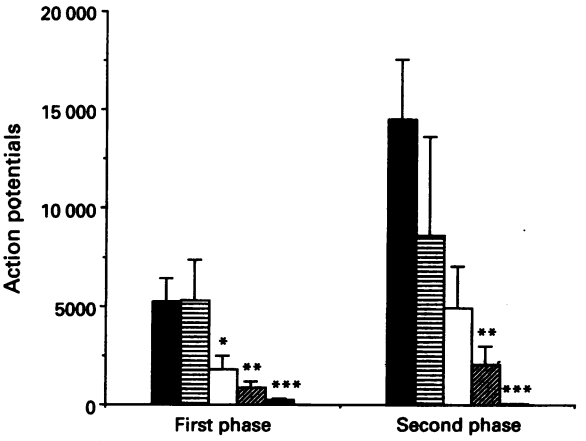

Figure 8 N^6-CPA the formalin-evoked responses of nociceptive neurones. Effects of N^6-CPA, given at 0.01 (▤), 0.1 (▭), 1 (▨) and 5 μg (□) on the first and second phase of the formalin-evoked response compared to controls (■); $n=5$ for each dose of N^6-CPA which was given 20 min before the formalin injection.

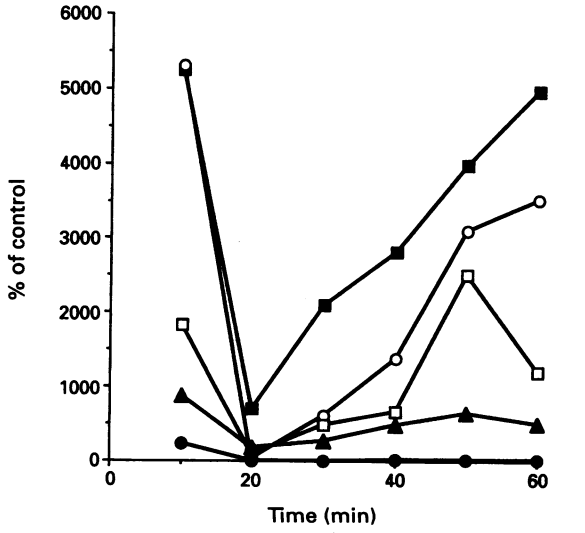

Figure 9 The mean effects of N^6-CPA on the formalin-evoked response plotted against time, with five neurones studied for each dose. The standard errors have been left off for clarity. The first phase (the first 10 min) and the second phase can clearly be observed in the control plot. The effects of N^6-CPA 0.01 (○), 0.1 (□), 1 (▲) and 5 μg (●) on the first and second phase, given as a 20 min pretreatment before formalin, compared to controls (■) are shown. The increasing duration of the effects of N^6-CPA with increasing doses can be seen.

Discussion

These electrophysiological results extend the evidence for a role of adenosine in the control of nociception at the spinal cord level (see Sawynok & Sweeney, 1989; Salter *et al.*, 1993) and support the importance of the A_1 receptor in these events.

A_1 receptor-mediated antinociception has been reported with behavioural tests (Sosnowski *et al.*, 1989; Karlsten *et al.*, 1990; Malmberg & Yaksh, 1993). In our studies, the A_1 agonist, N^6-CPA and the non-specific agonist, 2-Cl-Ado were first tested on the acute electrically evoked Aβ-, Aδ- and C-fibre-evoked activity on deep dorsal horn neurones. Both N^6-CPA and 2-Cl-Ado inhibited C-fibre-evoked activity, post discharge and wind-up. N^6-CPA potency inhibited these responses in a dose-dependent manner, whereas 2-Cl-Ado was weaker and produced sub-maximal inhibitions. With both agonists the low-threshold Aβ-evoked activity showed no change, indicating specific effects on nociception. In direct contrast to the other measures, the Aδ-fibre-evoked activity was facilitated by both agonists. The inhibitory effects of these agonists mirrored the facilitation of the Aδ-fibre-evoked activity, in terms of doses, maximal effects and time course.

With 2-Cl-Ado, a ceiling effect was seen for both the facilitations and inhibitions so it was not possible to quantify the dose-ratios. However, N^6-CPA was clearly more potent and efficacious, in agreement with general findings with these agonists. The doses of 2-Cl-Ado chosen would appear to be near the top of the dose-response range but as the maximal inhibitory effects of 2-Cl-Ado were less than those of N^6-CPA (indicative of a partial agonist effect, or effects of 2-Cl-Ado not restricted to the A_1 receptor) the dose-response curve was not extended, and N^6-CPA chosen for further testing. The selectivity and order of potency of these agonists is predictable from their A_1 receptor affinities (Jacobson *et al.*, 1992; Fredholm *et al.*, 1994).

The disparate effects of the agonists on these acute electrically evoked responses of the neurones is not likely to be due to differential penetration to anatomical targets since the doses and time-course of the effects, both inhibitory (C-fibres, wind-up and post-discharge) and facilitatory (Aδ) were the same. More superficially located neurones were not more sensitive than deeper cells, in keeping with a previous study, using opioids with a wide range of lipsolubilities given by the same route (Dickenson *et al.*, 1990).

The effects of both agonists were reversed to varying extents by theophylline. Reversal of the maximal inhibitory effects of the two agonists with 1000 μg theophylline was significant but not always complete which may reflect the fact that theophylline in a low affinity A_1 receptor antagonist (Fredholm *et al.*, 1994). Theophylline alone tended to enhance the C-fibre-evoked responses, post-discharge and wind-up of the neurones, indicative of an antagonism of tonic or evoked release of endogenous adenosine. However, phosphodiesterase inhibition as well as both A_1 and A_2 receptor antagonism (Williams, 1991; Fredholm *et al.*, 1994) cannot be ruled out as an explanation for these results. The antagonist 8(*p*-sulphophenyl) theophylline had no significant effect on the acute measured responses. Further studies with more selective antagonists would be useful in verification of the role of the A_1 receptor but are hampered by the lack of selective water-soluble compounds. The A_{2a} agonist (CGS-21680) produced nonselective inhibitions of the evoked activity including the low threshold Aβ responses, in contrast to the selective inhibitory effects of A_1 agonists on activity related to nociception.

In contrast to these inhibitory effects of both N^6-CPA and 2-Cl-Ado we observed concurrent facilitations of the Aδ-fibre-evoked firing of deep dorsal horn neurones which mirrored the inhibitions. Since the A_1 receptor is inhibitory, the observed facilitations of the Aδ response of the deep dorsal horn neurones could be due to a postsynaptic disinhibitory action. Stimulation of Aδ-fibres in the dorsal column causes a depression of nociceptive transmission in the spinal cord *in vitro* (Baba *et al.*, 1994). Interneurones in the substantia gelatinosa showed an initial excitatory post synaptic potential (e.p.s.p.) followed by fast and/or slow inhibitory post synaptic potential (i.p.s.p.) to this stimulation. If A_1 receptors were associated with these inhibitory interneurones, activation of the A_1 receptor would decrease inhibitions which could then result in disinhibition of the pathways transmitting Aδ information. The consequences of this A_1-induced facilitation for spinal nociception will depend on whether the Aδ responses represent high or low threshold inputs (see Baba *et al.*, 1994). The latter would appear more likely since spinal A_1 receptor agonists are antinociceptive in behavioural studies (Karlsten *et al.*, 1990; Malmberg & Yaksh, 1993).

Interestingly, theophylline and 8-pSPT did not reverse the facilitatory effects of the agonists on Aδ-evoked activity. The inability of these antagonists to reverse the effects of the agonists on the Aδ-evoked responses may indicate possible different adenosine receptor control of different components of evoked nociceptive activity. Another possibility in addition to disinhibition, is the A_3 receptor which is present in the CNS and in the rat, is insensitive to these xanthine antagonists (Linden, 1994). Thus it is conceivable that putative A_3 receptor-mediated effects of the agonists could underlie these facilitatory events. The absence of information on spinal cord location of this receptor, as well as a lack of characterized A_3 receptor antagonists hampers the study of its possible roles.

We found that N^6-CPA inhibited both phases of the formalin response, but that lower doses tended to be selective for the second phase. Other studies have shown that another A_1 agonist, N^6-[L-2-phenylisopropl]-adenosine (L-PIA) inhibits the second, but not the first phase of the formalin evoked response (Malmberg & Yaksh, 1993). This difference between our studies and this behavioural study may be due to the fact that L-PIA is a less potent agonist (Fredholm *et al.*, 1994) and that reductions in motor function, A_1 or A_2 receptor-mediated, (Sosnowski *et al.*, 1989; Karlsten *et al.*, 1990) prevented the doses from being increased which could have revealed first phase effects.

The formalin test was more sensitive to N^6-CPA than the acute electrically evoked activity, in keeping with results with other antinociceptive agents in this model (Chapman & Dickenson, 1993). Our effective doses of N^6-CPA were generally less than doses of L-PIA found to be effective in behavioural studies of the formalin response (Malmberg & Yaksh, 1993). We needed somewhat higher doses than those reported for acute behavioural measures (Karlsten *et al.*, 1990) to inhibit the acute evoked neuronal responses and so used high doses of theophylline to reverse these effects. This difference between the effective doses in acute studies may well result from the suprathreshold nature of the C-fibre stimulation we used as opposed to behavioural tests where thresholds are measured.

The effects of N^6-CPA on formalin-induced activity are entirely predictable from its effects on the electrically evoked activity. The overall prolife of the agonists in reducing the NMDA-mediated wind-up, post-discharges and second phase of the formalin response (Dickenson, 1994a; Haley *et al.*, 1990; Hunter & Singh, 1994) and also the facilitation of the Aδ responses is suggestive of postsynaptic A_1 receptor locations. Allodynia, touch-evoked nociception, has also been shown to be NMDA receptor-mediated (Yaksh, 1989) and modulated by L-PIA (Sosnowski & Yaksh, 1989). It is therefore highly likely that one mechanism behind our results is inhibition of excitatory interneurones in NMDA polysynaptic nociceptive pathways by activation of the A_1 receptor. However, the equivalent inhibitions of the non-NMDA-mediated acute C-fibre responses and the first early phase of the formalin response indicates further presynaptic actions, akin to opioids (Dickenson, 1994b).

There is evidence for a heterogeneous location of spinal A_1 receptors. They have been found on interneurones (Goodman & Snyder, 1982; Geiger *et al.*, 1984; Choca *et al.*, 1988) although kainic acid destruction of intrinsic neurones decreased spinal binding by only 33% (Geiger *et al.*, 1984) indicative of a

large proportion of presynaptic sites. Recent evidence from Li & Perl (1994) demonstrates that adenosine can have effects on both primary afferent neurones as well as interneurones, in keeping with the interpretation of our results.

The sites and mechanisms behind adenosine release are unclear. There is evidence for a presynaptic release (Holton & Holton, 1954; Holton 1959; Sweeney *et al.*, 1989) and small neurones in dorsal root ganglia have been shown to contain adenosine deaminase (Nagy *et al.*, 1984; Nagy & Daddona, 1985). Stimulation of low-threshold Pacinian afferents by vibration (Hunt, 1961) causes e.p.s.ps and i.p.s.ps in dorsal horn neurones (Hongo *et al.*, 1968; Salter & Henry, 1987) which are mediated by adenosine (Salter & Henry, 1987). This latter study, although supporting the idea of release from primary afferent neurones suggests low threshold fibres but not nociceptive fibres as the source. However, there is evidence for postsynaptic release mechanisms elsewhere in the CNS. Adenosine release in the cortex can be produced via AMPA and NMDA receptor activation (Hoehn & White, 1990; Craig & White, 1993). Studies (Craig & White, 1992; Manzoni *et al.*, 1994) have shown that adenosine may exert a negative feedback causing presynaptic depression in the cortex and hippocampus following postsynaptic NMDA receptor induced release. Our results indicate that adenosine acting at the A_1 receptor has an important role in indirectly controlling spinal NMDA dependent nociceptive pathways as well as other antinociceptive influences. It is therefore possible that adenosine can also function as a feedback mechanism in the spinal cord.

Overall, the present results further extend the scope of spinal adenosine A_1 receptor actions. It would appear that there could be multiple sites of action and mechanisms of release of adenosine, including release from and influences on both afferents and intrinsic neurones. A possible relatively selective control of NMDA-mediated events is suggested by the greater inhibitions of wind-up and post-discharge responses of the neurones. Future studies with other models of nociception and the development of selective adenosine agents suitable for *in vivo* approaches will shed further light on the role of this purine.

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