Classification of adenosine receptors mediating antinociception in the rat spinal cord

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1 Analogues of adenosine were injected intrathecally into rats implanted with chronic indwelling cannulae in order to determine a rank order of potency and hence characterize adenosine receptors involved in spinal antinociception.

2 In the tail flick test L-N⁶-phenylisopropyl adenosine (L-PIA), cyclohexyladenosine (CHA) and 5'-N-ethylcarboxamide adenosine (NECA) produced dose-related antinociception which attained a plateau level. NECA and CHA also produced an additional distinct second phase of antinociception. D-N⁶-Phenylisopropyl adenosine (D-PIA) and 2-chloroadenosine (CADO) had very little antinociceptive activity in this test. The rank order of potency in producing the plateau effect was L-PIA > CHA > NECA > D-PIA = CADO, while that for the second phase of antinociception was NECA > CHA.

3 Pretreatment with both theophylline and 8-phenyltheophylline (8-PT) antagonized antinociception produced by CHA, with 8-PT being at least an order of magnitude more potent than theophylline. Both antagonists produced a significant hyperalgesia in the tail flick test. L-PIA and CHA also produced methylxanthine-sensitive antinociception in the hot plate test.

4 These results suggest that activation of A₁-receptors in the spinal cord can produce antinociception. Activation of A₂-receptors may produce an additional effect, but the relative activity of CHA in this component of activity is unusual.

Introduction

The existence of two distinct adenosine receptors has been proposed. The original classification was based on opposing effects of adenosine on adenylate cyclase (Van Calker *et al.*, 1979). Activation of A₁-receptors produced a decrease in adenylate cyclase activity and a decrease in intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) whereas activation of A₂-receptors produced an increase in such activity. A further differentiation of these receptors has been made on the basis of the stereoselectivity of L-N⁶-phenylisopropyl adenosine (L-PIA) and D-N⁶-phenylisopropyl adenosine (D-PIA) as these differ by at least an order of magnitude in potency at A₁-receptors, but show weak stereoselectivity at A₂-receptors (Smellie *et al.*, 1979). More recently, it has been proposed that the rank order of potency of a number of analogues in addition to D-PIA and L-PIA is useful for the classification of A₁- and A₂-receptors (Stone, 1985; Burnstock & Buckley, 1985). This latter differentiation is significant because not all adenosine receptor effects are necessarily linked to adenylate cyclase.

Purines such as adenosine and its analogues have been shown to produce antinociception in the tail flick and hot plate tests following both systemic (Vapaatalo *et al.*, 1975; Holmgren *et al.*, 1983) and central (Yarbrough & McGuffin-Clineschmidt, 1981; Post, 1984) administration. In each case, antinociception was reversed by methylxanthines such as caffeine and theophylline suggesting the involvement of specific adenosine receptors. Antinociception produced by the systemic injection of PIA is stereoselective for the L-isomer (Ahlijanian & Takemori, 1985), implying the involvement of an A₁-receptor in this effect. However, 5'-N-ethylcarboxamide adenosine (NECA), which is generally one of the most potent agonists at A₂-receptors, is active following intrathecal injection (Post, 1984). In the present study, we have administered a number of adenosine analogues intrathecally to determine a detailed rank order of potency in producing antinociception in the tail flick test. The effects of two adenosine antagonists, theophylline and 8-phenyltheophylline (8-PT) on antinociception produced by these analogues and on baseline latencies were also studied.

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Methods

General

Male Sprague-Dawley rats (325–350 g), obtained from Canadian Hybrid Farms, Kentville, Nova Scotia, were anaesthetized with halothane and implanted with 7.5 cm chronic indwelling cannulae in the spinal subarachnoid space as described previously (Sawynok *et al.*, 1984). After surgery, animals were housed individually to prevent chewing of the cannulae. A 5–7 day recovery period was allowed, after which rats were used for 4–6 experiments with at least 2 days between trials. When tested specifically at the beginning and end of such a series of experiments, there was not an appreciable difference in responsiveness to adenosine analogues, indicating that repeated use was justified. Animals were randomly allocated into groups.

Noiceptive testing and quantitation of threshold changes

Noiceptive testing was performed using an automated tail flick apparatus (Ugo Basile, Italy). In tests for antinociception, baseline latencies were 2–3 s. A 10 s cut-off time was imposed and if animals had not responded by then, a 10 s value was assigned. In hyperalgesia experiments, the light intensity was adjusted so that baseline values were between 7 and 12 s. Previous studies have demonstrated that hyperalgesia

in this test is measured much more readily under such conditions (Sawynok *et al.*, 1984). In the hot plate test (plate temperature $50 \pm 0.5^\circ\text{C}$), baseline values were between 10 and 20 s and a cut-off time of 60 s was imposed.

For tail flick experiments, rats were accommodated in plastic boxes which allowed access to the cannula for intrathecal injections for the entire experiment. Following the determination of a baseline latency, drugs were injected intrathecally in a volume of $10\ \mu\text{l}$ and flushed in with a further $10\ \mu\text{l}$ of saline (cannula volume 7–8 μl). Nociceptive thresholds were determined at 15 min intervals for 60 min following injection of adenosine analogues. In hot plate experiments, rats were placed in plastic boxes only for the intrathecal injections.

Antinociception was quantitated using an Antinociceptive Index (AI) score. This was calculated by summing differences between each nociceptive value and the baseline value before injection for the 15, 30, 45 and 60 min determinations following intrathecal injection (approximates area under the time-response curve), i.e. $\text{AI} = \sum (\text{nociceptive threshold value} - \text{baseline value})$ s. When effects on baseline were determined, cumulative changes in latency were expressed as positive or negative deflections from the baseline value. A cumulative negative deflection significantly different from that produced by the vehicle indicates hyperalgesia.

Statistics were calculated using analysis of variance followed by Student-Newman-Keuls' test.

Figure 1 Time course of antinociception produced by intrathecal injections of (a) 5'-N-ethylcarboxamide adenosine, (b) L-N⁶-phenylisopropyl adenosine and (c) cyclohexyladenosine in the tail flick test. Doses in nmol indicated on figure. Injections were made at arrows following a baseline determination. Values are means for $n = 5-9$ rats, with s.e.mean < 1.0 s.

Drugs

The D- and L-isomers of phenylisopropyl adenosine, cyclohexyl adenosine (CHA) and 5'-N-ethylcarboxamide adenosine were obtained from Research Biochemicals Incorporated, Massachusetts, 2-chloroadenosine (CADO) and aminophylline (theophylline, ethylenediamine) from Sigma, St Louis, and 8-phenyltheophylline (8-PT) from Aldrich Chemical Company, Milwaukee.

8-PT was dissolved in 0.02N NaOH. In these experiments, comparisons were made with control animals treated with this vehicle. All other drugs were dissolved in normal saline.

Results

The adenosine analogues NECA, L-PIA and CHA produced dose-related antinociception in the tail flick test following intrathecal injection of 0.25–10 nmol of drug (Figure 1). The highest dose given corresponds to

an injection of 10 μ l of a 1 mM solution. Elevations in tail flick latency were determined up to 60 min, but clearly with higher doses could persist for longer (Figure 1). When results from the tail flick test were expressed in the form of an index, dose-response curves displayed a plateau level of activity (Figure 2). NECA and CHA produced a biphasic effect in the tail flick test. This manifested as an abrupt discontinuity in the dose-response curve illustrating indexes, and a sudden increase in tail flick latency over a small dose increment in the time course curves (Figure 1). The relative potency of analogues in producing the plateau effect was L-PIA > CHA > NECA > D-PIA = CADO, while the order of potency in producing the second phase of antinociception was NECA > CHA > L-PIA. (The existence of the second phase with L-PIA is questionable.)

Pretreatment with theophylline and 8-phenyltheophylline (8-PT) antagonized the antinociception produced by CHA (1 nmol and 5 nmol) in the tail flick test in a dose-dependent manner (Figures 3 and 4). As an antagonist, 8-PT was 15–40 times more potent

Figure 2 Dose-response curves for adenosine analogues in the tail flick test. (Δ --- Δ) L-N⁶-phenylisopropyl adenosine (L-PIA), ($\circ$ — $\circ$) cyclohexyladenosine (CHA), ($\bullet$ — $\bullet$) 5'-N-ethylcarboxamide adenosine, (∇ --- ∇) D-PIA, ($\square$ — $\square$) 2-chloroadenosine. Values are means, with vertical lines indicating s.e.mean of $n = 5$ –12 rats, except for CHA 1 and 5 nmol where $n = 30$ and 15, respectively. Antinociceptive index is the sum of tail flick readings minus the baseline value prior to injection for determinations at 15, 30, 45 and 60 min following intrathecal injection.

Figure 3 Dose-related antagonism of the antinociceptive effect of cyclohexyladenosine (CHA; 1 nmol) produced by theophylline (TH) and 8-phenyltheophylline (8-PT). In (a), (●) saline (Sal), theophylline (○) 11.8 nmol, (×) 118 nmol injected at first arrow 15 min prior to a tail flick reading and injection of CHA 1 nmol at the second arrow. In (c), (●) 0.02 N NaOH, 8-PT (○) 0.12 nmol, (×) 1.18 nmol injected at first arrow. In (b) and (d), the same data are expressed as an index. * $P < 0.05$, ** $P < 0.01$ compared to saline or NaOH pretreated controls. Values are mean with vertical lines indicating s.e.mean of number of rats indicated in columns.

than theophylline (Figure 4). Both theophylline and 8-PT produced significant hyperalgesia over a 60 min time course in the tail flick test when baseline values were between 8–10 s (Figure 5). Hyperalgesia 15 min after injection of methylxanthines was more variable in antinociception experiments where baseline latencies were 2–3 s (Figure 3).

The antinociceptive effects of some of the adenosine analogues were also determined in the hot plate test.

Results are summarized in Figure 6a. Both L-PIA and CHA produced antinociception, while very little activity was observed with D-PIA. As with the tail flick test, a plateau of activity was observed with L-PIA between 1 and 5 nmol. Curiously, CHA at a dose which produced the second phase of antinociception in the tail flick test did not produce an effect greater than this plateau level. Both theophylline and 8-PT antagonized antinociception in the hot plate test

Figure 4 Relative potency of 8-phenyltheophylline and theophylline in antagonizing the antinociceptive effect of cyclohexyladenosine (CHA) 1 and 5 nmol in the tail flick test. Data were converted to the index form as shown in Figure 3 (b and d), then expressed as a percentage of the control (vehicle pretreated) group. (●, ▲) 8-Phenyltheophylline; (○, △) theophylline; (●, ○; solid line) evaluated against CHA 1 nmol (mean control Antinociceptive Index 6.4–9.6, see Figure 3), (▲, △; broken line) evaluated against CHA 5 nmol (mean control Antinociceptive Index 15.4–17.8). Values are mean with vertical lines indicating s.e.mean of $n = 5$ –10 rats.

(Figure 6a). In this test, the negative cumulative change in baseline produced by theophylline and 8-PT did not differ from that produced by saline (Figure 6b). In addition to producing antinociception, CHA also produced an impairment of motor activity (a readily observable flaccidity) in the hind paws at the highest dose tested (5 nmol). This effect was not seen with any other analogue in the dose range tested. Motor impairment had a longer latency than antinociception, in that it did not develop until 30 min after injection, while antinociception was apparent 15 min after injection.

Discussion

The present results suggest that activation of both A_1 - and A_2 -receptors can produce spinal antinociception as assessed by the tail flick test. The order of potency of adenosine analogues in producing the response which attains a plateau level was L -PIA > CHA > NECA > D-PIA = CADO. This is similar to the rank order of potency of analogues in displacing [3 H]-CHA from A_1 binding sites in the dorsal horn of the spinal cord (L -PIA > CHA > NECA > CADO > D-PIA) (Geiger *et al.*, 1984), as well as the generalized rank order of potency in other A_1 systems (L -PIA > D-PIA and L -PIA, CHA > NECA) (Daly, 1982; Burnstock & Buckley, 1985), suggesting an involvement of A_1 -receptors in the plateau effect. The presence of A_1 -

Figure 5 Effect of (a) theophylline (TH) and (b) 8-phenyltheophylline (8-PT) on the tail flick response. In (a) and (b) (●---●) saline (SAL) or vehicle (0.02 N NaOH), (○---○) theophylline or 8-PT. In (c), data are expressed as a cumulative change in latency from the mean of baseline readings prior to injection of the methylxanthine at the arrow. A significant negative change (open columns) indicates hyperalgesia. Values are means with vertical lines indicating s.e.mean of number of rats indicated in columns. * $P < 0.05$, ** $P < 0.01$ compared to (a) mean baseline prior to injection at arrow, (c) corresponding saline- or vehicle-treated controls.

Figure 6 Antinociceptive effect of D-N⁶-phenylisopropyl adenosine (D-PIA), L-PIA and cyclohexyladenosine (CHA) in the hot plate test in the absence and presence of theophylline (TH) or 8-phenyltheophylline (8-PT). Doses are indicated in nmol. The effects of antagonists alone on hot plate latency are indicated in (b) as a cumulative change from baseline latencies over a 60 min period following injection. Values are means with vertical lines indicating s.e.mean of number of rats indicated in columns. * $P < 0.05$, ** $P < 0.01$ compared to corresponding control.

receptors in the dorsal horn of the spinal cord has been demonstrated in both autoradiographic (Goodman & Snyder, 1982) and binding studies (Geiger *et al.*, 1984; Choca *et al.*, 1985). There is also a distinct second

phase of antinociception in this test in which NECA is more potent than CHA. The prominence of the activity of NECA is similar to that observed in other A₂-receptor systems (Daly, 1982; Burnstock & Buckley, 1985) suggesting that the activation of A₂-receptors mediates this second component of action. Consistent with this notion are the observations that A₂ binding sites are present in the spinal cord (Choca *et al.*, 1985) while NECA (Aran *et al.*, 1985) but not L-PIA (Proudfoot, personal communication) potentiates the antinociceptive effect of intrathecal noradrenaline. However, CHA was the only other analogue which unequivocally produced this second component of activity in our study. This is unusual because CHA is not generally thought to have prominent activity at A₂-receptors (Daly, 1982; Burnstock & Buckley, 1985) although there may be exceptions to this in nervous tissue (Wu *et al.*, 1982). Curiously, at a dose which produced a prominent second phase of antinociception in the tail flick test, CHA did not produce an effect any greater than the plateau effect seen with L-PIA in the hot plate test. Whether the rank order of potency of adenosine analogues in other tests for nociception would be the same remains to be seen. It is likely that with the tail flick test, the site of action of purines is completely spinal because intracerebroventricular injection of adenosine analogues did not produce activity in the tail flick test even though activity in the hot plate test was observed (Yarbrough & McGuffin-Clineschmidt, 1981). In the hot plate test, the potential for an additional supraspinal action would need to be considered.

The classification of adenosine receptors in the present study relies on the rank order of potency of a number of analogues. Following intrathecal injection, the contribution of variable penetration of the blood-brain barrier by the various analogues is eliminated, indicating an advantage of the present system over systemic administration. However, the analogues used still differ in lipid solubility and presumably penetration to the site of action in the spinal cord, and these could contribute to the differing potencies observed. Nevertheless, there is a good general agreement with a variety of other *in vivo* and *in vitro* systems (Burnstock & Buckley, 1985) where such factors are functional and can potentially contribute to differences in potency.

The highest dose of CHA used in this study produced a motor impairment in the form of hind limb flaccidity. This effect was not seen with other analogues in the doses tested. However, motor impairment following intrathecal injection of NECA to mice has been observed (Post, 1984) suggesting this effect is not exclusive to CHA. Motor impairment could be dissociated from antinociception in that antinociception could be observed with lower doses and a shorter latency to onset. The longer time required for motor

impairment to occur may reflect a longer time to penetrate to motor neurone sites of action in the ventral horn of the spinal cord. Both A₁- and A₂-receptors are present in the ventral horn (Geiger *et al.*, 1984; Choca *et al.*, 1985) while adenosine and related purines can hyperpolarize both dorsal and ventral roots of the amphibian spinal cord by a theophylline-sensitive mechanism (Phillis & Kirkpatrick, 1978).

In the present study, 8-PT was at least an order of magnitude more potent than theophylline as an antagonist in blocking both the plateau and second component effect of adenosine analogues in the tail flick test, as well as in the hot plate test. 8-PT is more potent than theophylline in antagonizing effects of purines on cholinergic nerve terminals in the guinea-pig ileum (Griffith *et al.*, 1981) and on adrenergic nerve terminals in the rat vas deferens (Clanachan, 1981), effects classified as being mediated via A₁-receptors on the basis of the high degree of stereoselectivity for L-PIA compared to D-PIA (Paton, 1981). 8-PT is also more potent than theophylline at inhibiting adenosine receptor stimulated cyclic AMP production via the A₂-receptor (Smellie *et al.*, 1978). These antagonists are not specific for adenosine receptor subtypes and do not facilitate their classification (Daly, 1982). Nevertheless, their use enables adenosine receptors as a class to be implicated in a particular pharmacological effect.

Both theophylline and 8-PT produced significant hyperalgesia in the tail flick test. Hyperalgesia in this test has been observed previously following systemic (Sawynok, 1983) and intrathecal injection (Jurna, 1984) of theophylline and aminophylline. The occurrence of hyperalgesia suggests that there may be an ongoing tonic release of purines in the spinal cord which normally regulates nociceptive thresholds. This

possibility requires further investigation.

The mechanism by which adenosine analogues produce antinociception in the spinal cord is not clear. The binding of [³H]-CHA to A₁-receptors has been found to be unaffected by dorsal rhizotomy, neonatal capsaicin pretreatment and spinal cord hemisection, suggesting that A₁-receptors are not presynaptic to primary afferent nerve terminals or pathways descending from supraspinal structures (Geiger *et al.*, 1984). Consistent with this absence of receptors from primary afferent terminals is the observation that CHA (10⁻⁵ M) did not inhibit the release of substance P from slices of spinal cord in an *in vitro* superfusion system (Vasko *et al.*, 1986). Pretreatment with kainic acid reduced [³H]-CHA binding suggesting A₁-receptors may be located on interneurons in the spinal cord (Geiger *et al.*, 1984). The location of A₂-receptors within the dorsal spinal cord (Choca *et al.*, 1985) has not yet been determined. There are as yet no data implicating alterations in cyclic AMP levels in the spinal action of adenosine analogues, but this clearly requires examination. Although there is some evidence suggesting a role for changes in cyclic AMP levels contributing to the spinal effect of morphine in the tail flick test (Jurna, 1984), another study has disputed an involvement of cyclic AMP in nociception (Duggan & Griersmith, 1979). Both theophylline (Jurna, 1984) and 8-PT (Sweeney *et al.*, 1986) have been shown to inhibit the antinociceptive effect of morphine injected intrathecally, indicating that the role of purines in spinal antinociceptive mechanisms may be of considerable importance.

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