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Complex role of peripheral adenosine in the genesis of the response to subcutaneous formalin in the rat

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Abstract

When applied peripherally, adenosine has been shown to be pronociceptive in a number of animal and human models. Recent evidence has implicated adenosine as a significant mediator in the inflammatory process. In this study using rats, we have examined the effect of adenosine and of selective adenosine A_1 and A_2 receptor agonists and antagonists on the response to a subcutaneous injection of formalin into the rat hindpaw. Adenosine co-injected with formalin 0.5% significantly increased flinching in both phases in a dose-dependent manner. The highest dose of adenosine had no behavioral effect on its own. The adenosine A_2 receptor agonist 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamido adenosine hydrochloride (CGS-21680), at a dose of 1.5 nmol, increased flinching associated with 0.5% formalin injection but at higher doses produced depressant effects due to systemic absorption. The adenosine A_1 receptor agonist N^6-cyclohexyladenosine produced only systemic behavioral effects as determined by contralateral application. The flinching response to 2.5% formalin was significantly decreased by the adenosine A_2 receptor antagonist 3,7-dimethyl-1-propargylxanthine (DMPX). In contrast, 8-cyclopentyl-1,3-dimethylxanthine (CPT), the selective adenosine A_1 receptor antagonist augmented the response to 2.5% formalin. The non-selective adenosine receptor antagonist caffeine had no significant effect over a wide range of doses. In summary, exogenous adenosine enhances nociception in the formalin test, probably via a peripheral A_2 receptor-mediated action. Endogenous adenosine, acting at both A_1 and A_2 receptors, appears to be involved in the formalin-induced inflammatory response. This activation of adenosine A_1 and A_2 receptors may have opposing effects on nociceptive input.

Keywords: Formalin test; Antinociception; Adenosine; Methylxanthine; Pain mechanism

1. Introduction

The antinociceptive action of adenosine analogues is well established. The spinal administration of a number of adenosine receptor agonists produces antinociception in a variety of animal models of pain and both adenosine A_1 and A_2 receptors are implicated (reviewed by Sawynok, 1991). Antinociception following systemic administration of adenosine agonists appears to be manifest largely through activation of spinal receptors (Holmgren et al., 1986). In contrast, the activation of peripheral adenosine receptors has an algesic or pronociceptive effect. When administered by the intravenous (Sylvén et al., 1986, 1988a) or intracoronary route (Lagerqvist et al., 1990) in human subjects, adenosine is capable of producing chest pain characteristic of angina pectoris. Similar ischemic type pain occurs in the forearm with injection of adenosine into the brachial artery (Sylvén et al., 1988b). Direct application of adenosine to the human blister base produces pain (Bleehen and Keele, 1977). These pronociceptive actions of adenosine are blocked by methylxanthines, suggesting an effect on adenosine cell surface receptors.

Evidence has emerged for a peripheral hyperalgesic effect of adenosine in two animal models of pain. Taiwo and Levine (1990) have shown a hyperalgesic effect of adenosine in the rat paw-withdrawal pressure threshold reflex. A direct action on the primary afferent nociceptor through an adenosine A_2 receptor was

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implicated in this action. In mice, the first phase response to injection of dilute formalin into the paw has been shown to be augmented by adenosine A_2 receptor activation (Karlsten et al., 1992). These data suggest a role for adenosine in the development of hyperalgesia associated with phasic pain paradigms and a direct enhancement of chemically induced pain.

Adenosine receptors are present on inflammatory cells and adenosine appears to play a significant role during the inflammatory process (Cronstein, 1994). Tissue adenosine levels have been shown to be enhanced under conditions of ischemia and inflammation (Matherne et al., 1990; Cronstein, 1994). In view of the inferred presence of adenosine receptors on sensory neurons in mediating hyperalgesia and pronociception (see above), endogenous adenosine may well play a role in the genesis of inflammatory pain.

The formalin test provides a model of the behavioral response to a moderate, tonic, inflammatory pain. It has been suggested that the first phase of the formalin test in rodents results from direct chemical stimulation of nociceptors whereas the second phase involves peripheral inflammatory processes (Dubuisson and Dennis, 1977; Hunskaar and Hole, 1987; Tjølsen et al., 1992). While adenosine analogues have been demonstrated to enhance the first phase of this response (Karlsten et al., 1992), there is no information available on its effects during the second, tonic phase. The present study was undertaken to investigate the role of peripheral adenosine in the generation of pain under the inflammatory conditions associated with the second phase of the formalin test in rats by investigating the effects of exogenously administered adenosine receptor agonists, as well as endogenously generated adenosine by determining the effects of adenosine receptor antagonists.

2. Materials and methods

2.1. Animals

All experiments were conducted on male Sprague-Dawley rats (Charles River, Quebec, Canada) weighing between 100 and 150 g. The animals were housed in groups of 2–4 in the animal care facility and maintained on a 12 h light/dark cycle with rat chow and water available ad libitum. A minimum 24 h acclimatization period was allowed after shipment to the facility. On the day of testing, rats were removed from the animal care facility to the testing area at least 1 h prior to testing. All procedures were reviewed by the University Committee on Laboratory Animals and deemed to be in accordance with the Canadian Council on Animal Care Guidelines and IASP guidelines on the use of animals in pain research.

2.2. Formalin test

Rats were placed in a 30 cm cubic Plexiglas observation chamber for 10–15 min to accommodate to their surroundings, then removed for formalin administration. Under brief halothane anesthesia, the dorsum of the rat hindpaw was injected with 50 μl of dilute formalin/test solution using a 30 gauge needle. The rat was immediately placed in the observation chamber and within 2 min had recovered from the anesthesia, at which time nociceptive scoring started.

The rats were observed for nociceptive behavior in alternate 2 min bins, commencing 2 min post injection until 60 min post injection (total of 15 bins). Two animals were observed per trial, in alternate 2 min bins. Nociceptive behavior was quantified as the number of lifts or flinches of the affected limb during the observation bin. Such behavior could vary between a simple lift of the paw (not associated with locomotion) to a vigorous shaking of the limb, or it could be a rippling of back muscles associated with limb movement. Lifts or flinches were discrete and easily quantifiable. Formalin-induced flinching behavior has been shown to be more robust than the paw licking response and less affected by other behavioral influences (Wheeler-Aceto and Cowan, 1991).

2.3. Drugs and formalin concentrations

A dose-response characterization was performed initially to determine optimum formalin concentrations (Fig. 1). Where the test drug was anticipated to augment the response, 0.5% formalin (1:200 dilution of stock formalin solution, 37% formaldehyde, in 0.9% saline) was used for injection. Where antagonism of the response was anticipated, 2.5% formalin (1:40 dilution of stock formalin in 0.9% saline) was co-injected with the test drug.

Adenosine receptor agonists tested included adenosine, the adenosine A_1 receptor agonist N^6-cyclohexyladenosine and the adenosine A_2 receptor agonist 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS-21680). The adenosine agonist N^6-cyclohexyladenosine has an affinity for the adenosine A_1 receptor approximately 395-fold greater than for the adenosine A_2 receptor while CGS-21680 has a 173-fold greater affinity for the adenosine A_2 versus A_1 receptor (Collis and Hourani, 1993). Adenosine receptor antagonists used were 8-cyclopentyl-1,3-dimethylxanthine (CPT), with a 132-fold selectivity for the adenosine A_1 receptor (Bruns et al., 1986); 3,7-dimethyl-1-propargylxanthine (DMPX), with a 57-fold selectivity for the adenosine A_2 receptor (Seale et al., 1988); and the nonselective adenosine receptor antagonist, caffeine (Bruns et al., 1986).

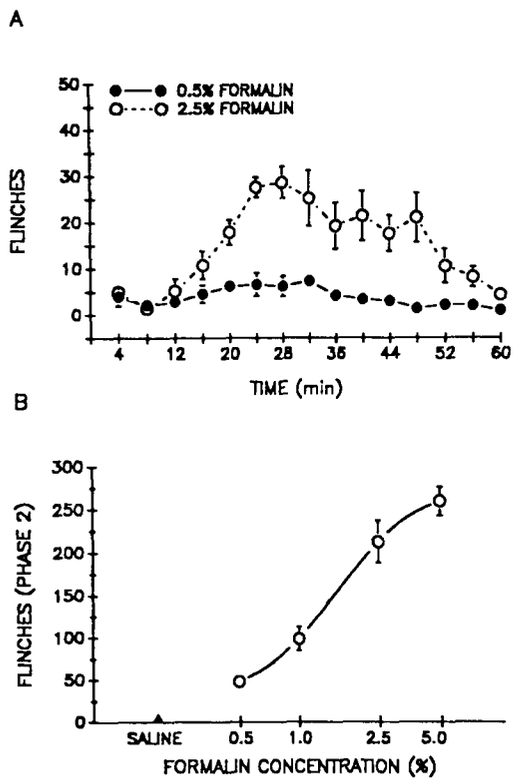

Fig. 1. A: Time course of flinching in response to subcutaneous injections of formalin concentrations of 0.5% and 2.5% at time zero. Each point represents cumulative flinches over the preceding 2 min bin. Each individual was observed on alternate 2 min bins beginning 2 min post-injection. B: Phase 2 response in rats (cumulative flinches in alternate 2 min bins between 14 and 60 min) seen with increasing formalin concentrations ($n = 5$–7 rats/group, $P < 0.05$ vs. saline for all concentrations tested). Values are means ± S.E.M. Where S.E.M. bars are not shown, they are within the symbol.

Drugs were prepared in saline or dimethyl sulfoxide (DMSO) and then added to a fresh formalin/0.9% saline solution to achieve final concentrations. CGS-21680 and N^6-cyclohexyladenosine required 10% DMSO while CPT required 20% DMSO. Appropriate concentrations of DMSO were added to control formalin injections.

2.4. Statistical analysis

Data collected over the 60 min of observation were divided into phase 1 (2–12 min, 3 bins) and phase 2 (14–60 min, 12 bins) as previously described (Dubuisson and Dennis, 1977; Wheeler-Aceto and Cowan, 1991). For group comparisons, the cumulative response in a given phase was analyzed using ANOVA followed by Dunnett's test.

2.5. Drug sources

Stock formalin solution, DMSO, caffeine, and adenosine were obtained from Sigma Chemical Co., St. Louis, MO, USA. The adenosine analogues (N^6-cyclohexyladenosine, CGS-21680, CPT, and DMPX) were obtained from Research Biochemicals, Natick, MA, USA.

3. Results

3.1. Formalin dose-response relationship

Formalin concentrations between 0.5% and 5.0% were tested to determine a dose-response relationship for phase 2 nociceptive responses (Fig. 1A and B). The injection of 2.5% formalin produced a two-phased response with a near-maximal phase 2 effect. Injection of 0.5% formalin was associated with minimal activation of the phase 2 response, although this was significantly greater than injection of saline alone ($P < 0.05$, Fig. 1B). Subsequent testing with adenosine and adenosine receptor ligands used either 0.5% or 2.5% formalin injections.

3.2. Effects of exogenous adenosine

Adenosine was co-injected with 0.5% formalin to determine if there was an augmentation of the phase 2 response. Adenosine had no significant effect until a dose of 50 nmol was injected. This significantly augmented the response to both phase 1 (data not shown) and phase 2 ($P < 0.05$, Fig. 2A and B). To determine if this response to exogenous adenosine also was seen with higher concentrations of formalin, a second formalin dose-response was constructed in the presence of adenosine 50 nmol (Fig. 2C). Although adenosine augments the phase 2 response to 0.5% formalin injection, this effect is less pronounced and no longer significant at higher concentrations (1.0% and 2.5%). Adenosine injection in the absence of formalin elicited no behavioral response (cummulative phase 2 response (mean ± S.E.M.) for adenosine = 4.7 ± 3.3 vs. saline = 4.7 ± 0.7, $n = 3$/group, NS).

3.3. Effects of selective adenosine receptor agonists

When co-injected with 0.5% formalin, the adenosine A_2 receptor agonist CGS-21680 at a dose of 1.5 nmol augmented the flinch response in phase 2 by 75% ($P < 0.05$, Fig. 3). At progressively higher doses of CGS-21680 flinching returned to control levels and then below this level (Fig. 3). The highest dose of CGS-21680 (50 nmol) was associated with marked motor and behavioral changes, including reduced exploration, reduced grooming, drooping eyelids, and flattened body posture. These behavioral effects and the associated reduction in flinching at the highest dose (50 nmol) were due to a systemic effect of the adeno-

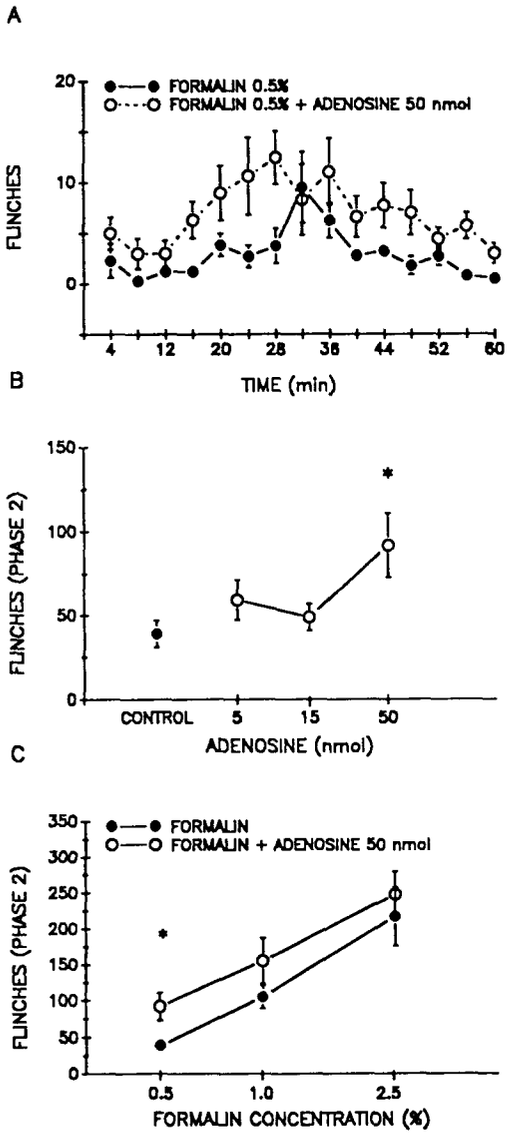

Fig. 2. A: Time course of flinching in response to 0.5% formalin alone and with 50 nmol adenosine co-injected. B: Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min) to 0.5% formalin injection (control) and co-injection of 0.5% formalin with increasing doses of adenosine ($n = 5$–7 rats/group, $^*P < 0.05$ vs. control). C: Phase 2 response to injection of increasing concentrations of formalin (0.5–2.5%) with or without 50 nmol of adenosine co-injected ($n = 5$–7 rats/group, $^*P < 0.05$ vs. formalin alone). Values are means ± S.E.M. Where S.E.M. bars are not shown, they are within the symbol.

sine A_2 receptor agonist since injection of 50 nmol CGS-21680 into the paw contralateral to the formalin injection site produced identical behavioral changes (Fig. 3B).

Observations in the first phase of the formalin test in mice (Karlsten et al., 1992) suggested a possible peripheral antinociceptive effect for adenosine A_1 receptor agonists. Because of this, the adenosine A_1 receptor agonist N^6-cyclohexyladenosine was tested in the presence of 2.5% formalin (Fig. 4). N^6-Cyclohexyladenosine produced a dose-related reduction in phase 2 activity, but this was associated with behavioral changes similar to those seen with the highest dose of CGS-21680. Contralateral injection of the same doses of N^6-cyclohexyladenosine produced identical behavioral and antinociceptive changes (Fig. 4), therefore a centrally mediated antinociceptive action cannot be excluded.

3.4. Effects of selective adenosine antagonists

The role of endogenous adenosine in phase 2 of the formalin test was investigated by co-injection of selective adenosine antagonists with a near-maximally effective concentration of formalin (2.5%). The adenosine A_2 receptor antagonist DMPX reduced phase 2 flinches by 32% ($P < 0.05$) (Fig. 5B). From the time course (Fig. 5A) it can be seen that most of the

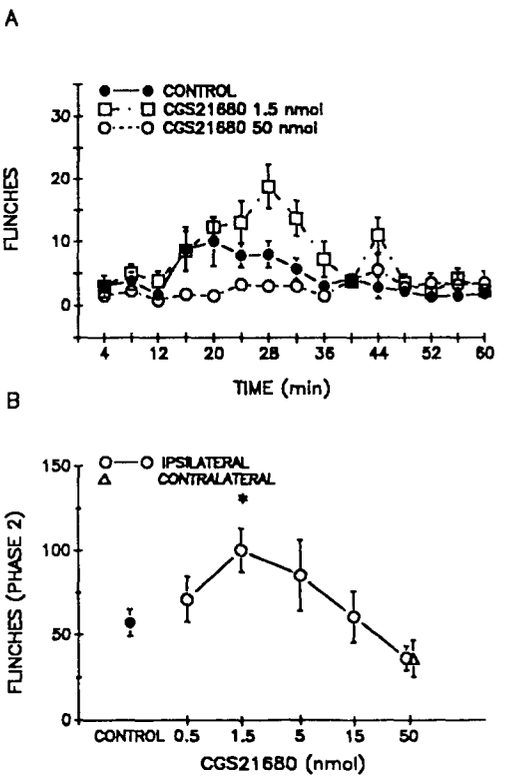

Fig. 3. A: Effects of co-injection of CGS-21680 on the time course of flinching in response to 0.5% formalin ($n = 5$–7 rats/group). B: Phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min) to 0.5% formalin injection (control) and co-injection of 0.5% formalin with increasing doses of CGS-21680 ($n = 5$–7 rats/group, $^*P < 0.05$ vs. control). Response to injection of the highest dose of CGS-21680 contralateral to 0.5% formalin also shown. Values are means ± S.E.M. Where S.E.M. bars are not shown, they are within the symbol.

reduction in flinching (39% control) occurred in the first half of phase 2, from 14 to 36 min, designated phase 2A (Fig. 5B, $P < 0.05$). Fig. 6 shows the response to 2.5% formalin co-injected with the adenosine A_1 receptor antagonist CPT. Flinching in phase 2A was increased by 61% ($P < 0.05$) in the presence of 15 nmol CPT. This increased response appeared to subside by the second half of phase 2 (Fig. 6A). We repeated the CPT doses in the presence of the minimally effective concentration of formalin (0.5%), but observed no significant drug effect at any dose tested (data not shown).

3.5. Effects of caffeine

We have recently shown that systemically administered caffeine produces antinociception in the rat formalin test (Sawynok et al., 1995) which potentially could result from peripheral adenosine A_2 receptor antagonism. In the presence of 2.5% formalin, locally administered caffeine had no significant effect on nociceptive behavior (Fig. 7).

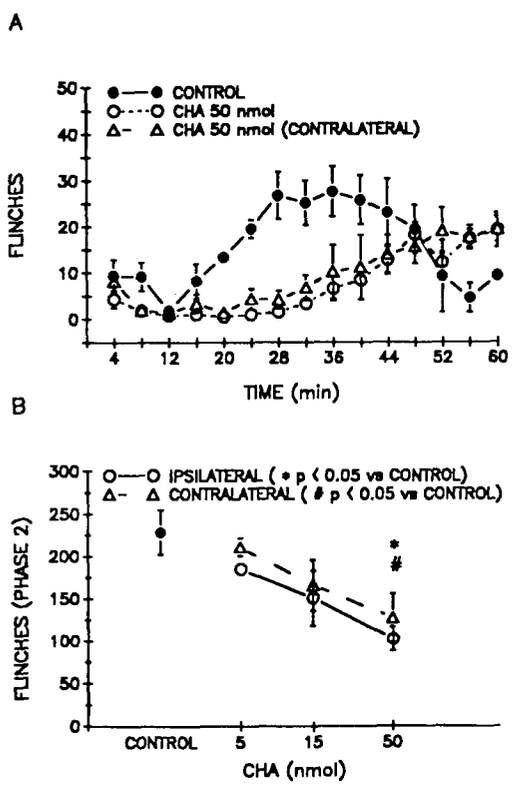

Fig. 4. A: Time course of the effect of N^6-cyclohexyladenosine (CHA), co-injected with formalin 2.5% or injected contralateral to formalin ($n = 5$–7 rats/group). B: Comparison of phase 2 response (cumulative flinches in alternate 2 min bins between 14 and 60 min) to 2.5% formalin injection (control), co-injection with CHA (ipsilateral) or following injection of CHA contralateral to 2.5% formalin ($n = 5$–7 rats/group). Values are means ± S.E.M. Where S.E.M. bars are not shown, they are within the symbol.

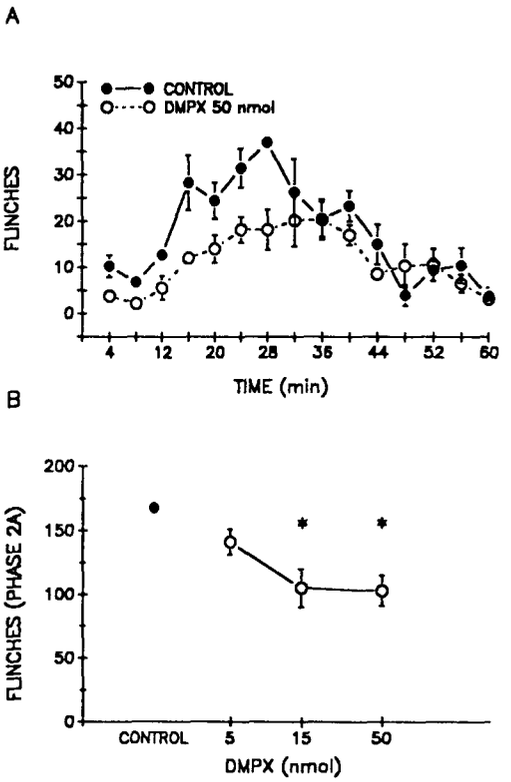

Fig. 5. A: Effects of co-injection of DMPX on the time course of flinching in response to 2.5% formalin ($n = 5$ rats/group). B: Phase 2A response (cumulative flinches in alternate 2 min bins between 14 and 36 min) to 2.5% formalin injection (control) and co-injection of 2.5% formalin with increasing doses of DMPX ($n = 5$ rats/group, $^*P < 0.05$ vs. control). Values are means ± S.E.M. Where S.E.M. bars are not shown, they are within the symbol.

4. Discussion

The results of this study suggest a dual role for adenosine in the periphery in the development of tonic inflammatory pain associated with the formalin test. The effect of endogenous adenosine under conditions of inflammation may involve coincidental activation of adenosine A_1 and A_2 receptor subtypes resulting in antinociceptive and pronociceptive effects, respectively. This role may coexist with antiinflammatory effects exerted on inflammatory cells such as neutrophils (Cronstein, 1994).

Adenosine augmented the flinching behavior associated with 0.5% formalin injection while eliciting no intrinsic effect on behavior. This pronociceptive effect appears to be due to adenosine A_2 receptor activation since the A_2 selective agonist CGS-21680 is also pronociceptive. This is consistent with the response to

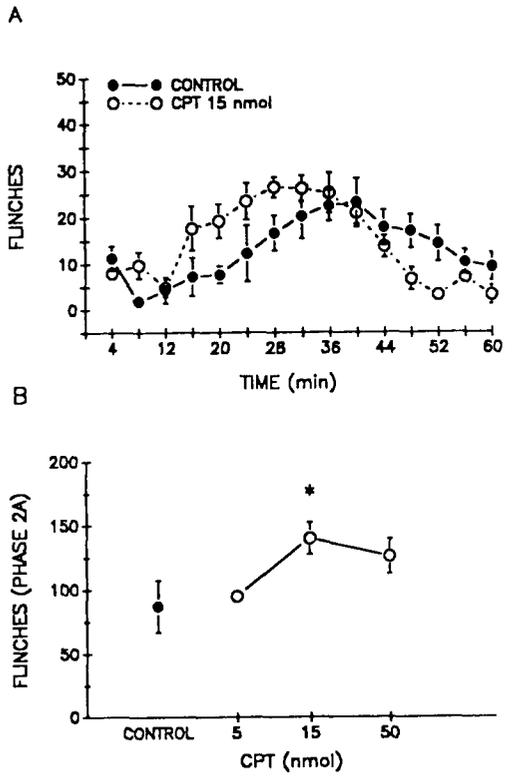

Fig. 6. A: Effects of co-injection of CPT on the time course of flinching in response to 2.5% formalin ($n = 5$–7 rats/group). B: Phase 2A response (cumulative flinches in alternate 2 min bins between 14 and 36 min) to 2.5% formalin injection (control) and co-injection of 2.5% formalin with increasing doses of CPT ($n = 5$–7 rats/group, $^{*}P < 0.05$ vs. control). Values are means ± S.E.M. Where S.E.M. bars are not shown, they are within the symbol.

adenosine analogues seen in the phasic paw-pressure withdrawal test (Taiwo and Levine, 1990) and the first phase of the formalin test in mice, as determined by the time spent licking or biting the injected paw (Karlsten et al., 1992). In both of these studies, adenosine A_2 receptor analogues were hyperalgesic or pronociceptive. It should be noted that both of these pain paradigms are considered to be a phasic stimulus while the second phase of the formalin test involves a tonic stimulus with an inflammatory component (Dubuisson and Dennis, 1977). When adenosine was administered in conjunction with a concentration of formalin producing a minimal nociceptive response (0.5%), flinching behavior throughout phase 2 was augmented by 137%. As the concentration of formalin was increased, the ability of adenosine to further augment the response became insignificant. This suggests a masked effect, possibly because endogenous adenosine is already being generated by the developing inflammatory response (Cronstein, 1994).

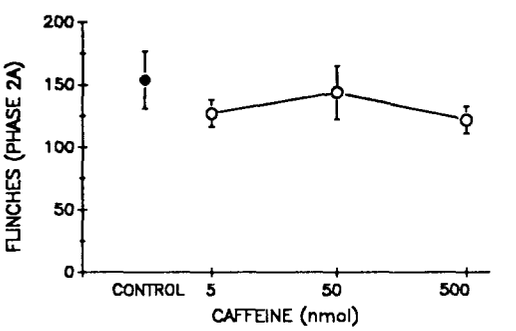

Fig. 7. Phase 2A response (cumulative flinches in alternate 2 min bins between 14 and 36 min) to 2.5% formalin injection (control) and co-injection of 2.5% formalin with increasing doses of caffeine ($n = 5$–7 rats/group). Values are means ± S.E.M.

Further evidence for a pronociceptive effect of adenosine A_2 receptor agonists is derived from observations with antagonists. Thus, the adenosine A_2 receptor antagonist DMPX can inhibit the inflammatory response generated by higher concentrations of formalin. The overall importance of endogenous adenosine to the inflammatory component of formalin pain may be limited as DMPX reduced flinching in phase 2A by a maximum of 39%. Other endogenous peripheral mediators of the inflammatory response are bradykinin, noradrenaline, histamine, 5-HT (5-hydroxytryptamine, serotonin), and eicosanoid products of the lipoxygenase or cyclo-oxygenase pathways of arachidonic acid metabolism (Dray et al., 1994). It may be that the real significance of the role of adenosine in inflammatory pain can only be appreciated in the presence of some of these other inflammatory mediators.

The hyperalgesic effect of peripheral adenosine A_2 receptor activation may be partially countered by an apparent analgesic effect mediated via adenosine A_1 receptor activation. Thus, when the system is stimulated by 2.5% formalin injection, adenosine A_1 receptor antagonism by CPT augments flinching. Interestingly, the augmentation of the nociceptive response was not seen when CPT was given in conjunction with 0.5% formalin. Again this suggests that only at the higher concentration of formalin is there a significant concentration of endogenous adenosine present. We were unable to detect an analgesic effect resulting from peripheral adenosine A_1 receptor activation using N^6-cyclohexyladenosine, as the central depressant effect of N^6-cyclohexyladenosine could not be separated from any peripheral analgesic effect it might have. The apparent opposing effects of adenosine on the second phase of the formalin test, which we have inferred using adenosine antagonists, was seen directly by Karlsten et al. (1992) using an agonist in the first phase in mice, and by Taiwo and Levine (1990) using the paw-withdrawal threshold test in rats. It should be noted that the analgesic effect attributed to peripheral adenosine A_1 receptor stimulation is in contrast to human data which suggests that peripheral A_1 receptor stimulation induces the pain associated with direct

intradermal injection of adenosine (Pappagallo et al., 1993).

The mechanism by which adenosine exerts simultaneous hyperalgesic and analgesic actions is not clear, but there is some evidence to suggest adenosine A_2 receptor-mediated hyperalgesia is mediated via a direct action on the primary affarent nerve terminal (Taiwo and Levine, 1990). Thus, the onset of action is rapid and comparable to other direct acting mediators, and interventions designed to remove indirectly generated responses do not alter the effect of adenosine (Taiwo and Levine, 1990). Methylxanthine-sensitive adenosine receptors are located on dorsal root ganglion cell bodies (Dolphin et al., 1986; MacDonald et al., 1986), providing support for the notion of a direct rather than indirect activation mechanism. The increase in adenosine-induced hyperalgesia in the presence of phosphodiesterase inhibition links the hyperalgesic action of adenosine to other directly acting hyperalgesic agents through the cAMP second messenger system (Taiwo and Levine, 1990). The mechanism involved in the adenosine A_1 receptor-mediated analgesia is less clear. In cultured dorsal root ganglion cell bodies, adenosine A_1 receptor-mediated inhibition of Ca^{2+} entry has been demonstrated (Dolphin et al., 1986; MacDonald et al., 1986). It is possible that adenosine A_1 receptor activation on the peripheral end of primary afferent nerve terminals results in inhibition of Ca^{2+} entry or cAMP production, or changes in inositol-1,4,5-trisphosphate generation. The fact that the dose of adenosine which enhanced the formalin response was not itself algesic suggests that in this model adenosine acts to either sensitize the nociceptors to the effects of other algesic substances or requires the nociceptor to be sensitized to exert its effects. This would be consistent with adenosine exerting a low level of stimulation of adenylate cyclase which is magnified in the presence of other direct acting agents (see above).

Caffeine produces analgesia in both human and animal nociceptive trials (Sawynok and Yaksh, 1993). In view of the peripheral pronociceptive actions of adenosine A_2 agonists (Taiwo and Levine, 1990; Karlsten et al., 1992), it was postulated that antagonism of peripheral adenosine A_2 receptors might reflect part of the mechanism of caffeine-induced analgesia (Sawynok and Yaksh, 1993). However, despite the analgesic activity of caffeine in the formalin test following systemic administration, no analgesic effect was seen following local administration of caffeine using 5% formalin (Sawynok et al., 1995) or 2.5% formalin (this study). The inference of adenosine A_1 receptor activation having an analgesic effect which can oppose the pronociceptive effect of adenosine A_2 receptor activation may provide an explanation for the apparent lack of effect of caffeine, a nonselective adenosine receptor antagonist, when administered peripherally. The analgesic efficacy of caffeine appears to result primarily from interactions at central sites (Sawynok et al., 1995).

It is difficult to separate sedative and locomotor effects of adenosine analogues from local effects on nociception. Higher doses of both CGS-21680 and N^6-cyclohexyladenosine produced definite systemic effects (sedation, decreased locomotor activity and flattened posture) that were apparent within 2–4 min of injection. This rapid appearance of systemic behavioral phenomena following local injection of adenosine analogues has been reported by others (Karlsten et al., 1992). It is possible that combining the drug injection with formalin, and the presence of DMSO, may have enhanced systemic absorption of the drugs, contributing to the early-onset action of these agents.

In summary, we have demonstrated that endogenous adenosine plays a significant role in tonic, inflammatory pain as revealed by the formalin test. Adenosine may exert simultaneous pro- and antinociceptive effects via activation of adenosine A_2 and A_1 receptors respectively. In this model, peripheral administration of a selective adenosine A_2 receptor antagonist and an adenosine A_1 receptor agonist might produce effective analgesia, while a peripherally acting nonselective antagonist could be of limited benefit at this site.

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