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Adenosine A_1 receptor-mediated excitation of nociceptive afferents innervating the normal and arthritic rat knee joint

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1 We tested the hypothesis that adenosine excites nociceptive primary afferents innervating the knee joint.

2 Neuronal recordings were made from fine nerve filaments innervating the knee joint in rats anaesthetized with pentobarbitone. Drugs were injected close-arterially (i.a.) or into the articular space (i.art.). We studied normal and chronically inflamed arthritic joints, the latter 14–21 days after a single intra-articular injection of Freund's Complete Adjuvant, performed under halothane anaesthesia.

3 Adenosine injected i.a. caused delayed (approximately 10 s) excitation of the majority of polymodal C-fibre afferents, and had similar effects when injected directly into the joint.

4 Adenosine triphosphate (ATP) had biphasic effects on discharge, a fast (<1 s) excitation was followed by a delayed increase similar to that seen with adenosine.

5 The adenosine A_1 receptor agonists N^6-cyclopentyladenosine (CPA) and N-[(1S,*trans*)-2-hydroxypentyl] adenosine (GR79236) also excited the C-fibre afferents. The A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) antagonized the responses evoked by adenosine, CPA, and the delayed increase seen after ATP, indicating that excitation of the nociceptive afferents was mediated *via* adenosine A_1 receptors.

6 Adenosine and ATP evoked delayed excitatory effects of similar magnitude, regardless of whether or not the knee joint was chronically inflamed. The increased basal discharge observed in arthritic joints was unaffected by DPCPX, which implies that the increase in spontaneous activity associated with arthritis is unlikely to involve tonically released adenosine.

7 The results support the hypothesis that adenosine excites primary afferent nociceptive nerve terminals in the rat knee joint, an effect mediated by adenosine A_1 receptors. ATP, adenosine, and A_1 receptors may play a role in generating the peripheral nociceptive (pain) signal.

Keywords: Adenosine; A_1 receptors; pain; nociception; sensory neurones; arthritis

Introduction

Evidence from human and animal studies suggests that adenosine plays a complex role in the generation and modulation of peripheral nociception (Keil & Salter, 1996; Sawynok, 1998). Adenosine has been reported to cause pain in humans when it is applied to a blister base (Bleehan & Keele, 1977), injected intradermally (Pappagallo *et al*., 1993), or injected intravenously (Gaspardone *et al*., 1995); it is also associated with the pain of angina pectoris (Sylven, 1993). However, others report that adenosine is anti-nociceptive in, for example, neuropathic pain (Belfrage *et al*., 1995). Similarly, in animal studies adenosine is reported to cause both hyperalgesia and analgesia (Taiwo & Levine, 1990).

The complexity of adenosine's actions in nociception may result from activation of different adenosine, receptors, four of which have been cloned (A_1, A_{2A}, A_{2B} and A_3; Collis & Hourani, 1993), and a combination of indirect actions mediated *via* inflammatory cells, and direct actions on primary afferent nociceptors. For example, adenosine acts on A_3 receptors to cause pain indirectly *via* the release of histamine and 5-hydroxytryptamine from mast cells (Sawynok *et al*., 1997). It might also cause pain by directly exciting primary afferent nociceptors; adenosine-induced cardiac pain is considered to be mediated by a direct action on adenosine A_1 receptors located on cardiac afferents (Sylven, 1993). This suggestion is supported by the observation that neurones in dorsal root (sensory) ganglia, which are excited by ischaemia and thought to mediate the pain of angina, are excited by adenosine in the dog (Huang *et al*., 1995).

A major source of extracellular adenosine is the metabolism of ATP by ectonucleotidases (Gordon, 1986). We have recently shown that ATP excites nociceptive afferents innervating the rat knee joint (Dowd *et al*., 1998a): it evoked a biphasic excitation consisting of a rapid increase in discharge (mediated by P2X receptors) followed by a delayed excitation. The delayed increase did not occur when we used the stable ATP analogue, $\alpha\beta$-methylene-ATP, which is resistant to ectoATPase and therefore not rapidly metabolized to adenosine. This evidence suggested that adenosine might be responsible for the delayed increase in afferent discharge that we obtained with ATP. If so, this would support the view that adenosine causes pain by exciting primary afferent nociceptors.

The present study was undertaken to test the hypothesis that adenosine excites nociceptive primary afferents innervating the rat knee joint. We used selective agonists and antagonists to characterize the type of adenosine receptor(s) involved, and also investigated whether chronic adjuvant-induced inflammatory arthritis affected the responsiveness of knee joint afferents to adenosine in anaesthetized rats. A preliminary report on this work has been published (Dowd *et al*., 1998b).

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Methods

Experiments were licensed under U.K. Home Office regulations and performed on 11 normal and 13 arthritic male Wistar rats (mean body weight 360 g, range 270–450 g).

Induction of arthritis

Freund's Complete Adjuvant (0.10–0.15 ml of *Mycobacterium tuberculosis* 1 mg ml^{-1} in paraffin oil, Sigma) was injected intra-articularly into the left knee joint of rats under halothane anaesthesia (5% in oxygen). Animals were used for electrophysiological recordings 14–21 days post-induction, at which time a mild persistent unilateral arthritis was present and associated with swelling (30% increase in diameter) and hyperalgesia of the knee.

Surgical procedure

Animals were anaesthetized with pentobarbitone (60 mg kg^{-1} i.p., supplemented hourly with 6 mg i.v. *via* a cannula inserted into the right femoral vein). The trachea and right carotid artery were cannulated and arterial blood pressure was continuously monitored. Body temperature was maintained at 38°C by an automated heating blanket connected to a thermistor probe inserted into the rectum. A cannula was inserted into the lower abdominal artery through the right femoral artery for close arterial injection of drugs to the left knee joint.

Electrophysiological recordings

Afferent neural discharge was recorded from a branch of the medial articular nerve which was cut centrally to eliminate efferent neural activity. The methods used have been described previously (Grubb *et al*., 1991; Dowd *et al*., 1998a). Briefly, neural activity was recorded *via* bipolar extracellular wire electrodes, and recorded digitally on videotape. The neural signal was analysed off-line using a pulse-height voltage discriminator linked to a personal computer operating Spike2 software (CED, Cambridge, U.K.). Single action potentials, identified by the size and shape of the spike, were counted separately. The receptive fields of the afferents were identified by probing the knee joint capsule with a hand held plastic probe (tip diameter 1 mm; Von Frey threshold for receptor activation >40 g mm^{-2}). Conduction velocities were determined at the end of experiments.

Drug administration

Agonists were injected in a volume of 0.1 ml, washed in with 0.2 ml of saline (0.9% w/v sodium chloride), the i.a. injection being completed within 2 s. Repeatable responses to agonists were obtained before using antagonists. Antagonists were injected i.a. over 10 s (in a volume of 0.1 ml 100 g^{-1} body weight) at least 10 min before agonists were re-tested. In three normal animals, drugs were administered by intra-articular injection into the knee joint using a 26-gauge needle inserted through the infrapatellar ligament just beneath the patella.

Data analysis

The effect of a drug, or vehicle, injection was determined by comparing the action potential discharge frequency after drug injection with that in the 15 s pre-injection period. Data are expressed as the mean change in action potential frequency $\pm$s.e.mean. Differences between means were analysed statistically using the Mann-Whitney test and the null hypothesis rejected at the 0.05 level of probability.

Drugs

Adenosine, adenosine 5′-triphosphate disodium salt (ATP), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), N^6-cyclopentyladenosine (CPA), bradykinin and 8-methyl-N-vanillyl-6-nonenamide (capsaicin) were purchased from Sigma; 2-*p*-(2-carboxyethyl) phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride (CGS-21680) from RBI, and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium (PPADS) from Tocris. N-[(1S,*trans*)-2-hydroxypentyl] adenosine (GR79236) was a gift from GlaxoWellcome, Stevenage, U.K. All drugs were dissolved in phosphate buffered saline (PBS) except for capsaicin, which was dissolved in Tween 80 (10% v/v), ethanol (10% v/v) and PBS, and DPCPX which was dissolved in DMSO (8% v/v), 1 M NaOH (2% v/v), and PBS.

Results

Action potentials were recorded from a total of 61 afferent nerve fibres, 25 innervating normal knee joints in 11 rats, and 41 innervating chronically inflamed joints in 13 rats; 48% of afferents innervating normal joints and 61% of those innervating arthritic joints were C-fibre polymodal nociceptors (mean conduction velocity: 1.08 ± 0.17 m s^{-1}) which were excited by capsaicin (10 nmol i.a.) and mechanical stimulation of the joint capsule. The remaining 52% of afferents innervating normal joints and 39% innervating arthritic joints were Aδ-afferents (conduction velocity: 3.43 ± 0.54 m s^{-1}) which were excited by mechanical stimulation of the joint, but not by capsaicin.

Adenosine (370 nmol) evoked an increase in action potential discharge in 80% (8/10 afferents) of C-fibres and 25% (3/12 afferents) of Aδ-fibres innervating normal knee joints. It also increased activity in 77% (17/22 afferents) of C-fibres and 27% (3/11 afferents) of Aδ-fibres innervating arthritic knee joints (Figure 1). Although the responses were similar in both fibre types, all the results presented in this paper are from C-fibres as there were too few adenosine-positive Aδ-fibres to study systematically. The magnitude, onset latency and duration of the adenosine-evoked response did not differ significantly between normal and arthritic joints (Figure 2). The drug vehicle (PBS, 0.3 ml i.a.) had no effect on discharge in adenosine-sensitive afferents (mean increase 0.01 ± 0.01 impulses s^{-1}, $n=6$). There was no evidence of desensitization when successive doses of adenosine were administered at intervals of 15 min.

ATP (2000 nmol i.a.) evoked a biphasic increase in neural discharge consisting of a fast-onset response followed by a delayed or 'slow' excitation (see Figure 1). We have previously shown that the fast desensitizing component is mediated by P2X receptors (Dowd *et al*., 1998a), and it will not be discussed further in this paper. The slow component was evoked in 78% (7/9 afferents) of C-fibres and 10% (1/10 afferents) of Aδ-fibres innervating normal knee joints, and 83% (19/23 afferents) of C-fibres and 19% (3/16 afferents) of Aδ-fibres innervating arthritic joints. The magnitude and duration of the slow ATP-evoked response in C-fibres was not significantly different for arthritic joints, in comparison with normal joints, but the latency to onset was significantly longer in the arthritic joints (Figure 2). There was no evidence for desensitization of the slow response to ATP.

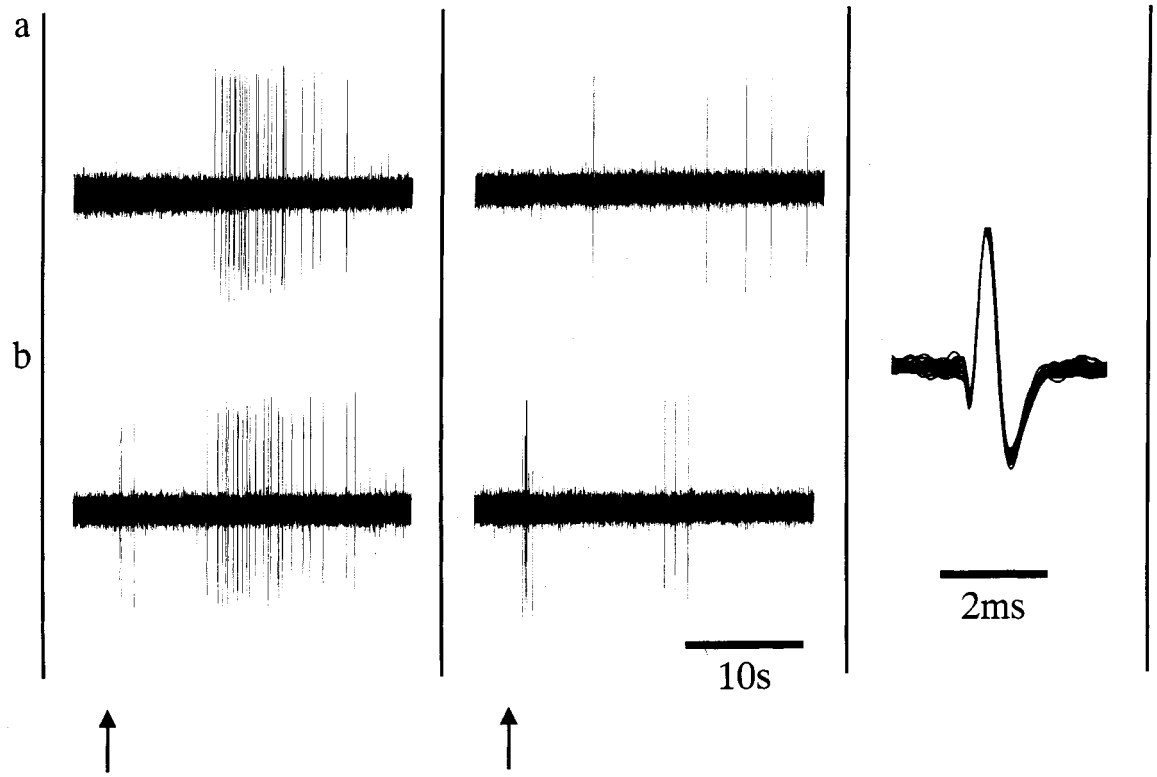


Figure 1 Oscilloscope traces showing the discharge evoked from a nerve filament innervating a normal knee joint by i.a. injection (at arrow) of (a) adenosine (370 nmol) and (b) ATP (2000 nmol). The first panel shows control responses, the second shows the responses 10–30 min after injection of DPCPX (3 μmol kg^{-1}). Note that the slow responses to adenosine and ATP are antagonized by DPCPX while the fast-onset response to ATP is not. The third panel shows the C-fibre that responded to adenosine and ATP.

In experiments during which adenosine and ATP were both tested, all the fibres that were excited by adenosine also responded with a delayed excitation to ATP (normal $n=8$ afferents; arthritic $n=22$ afferents). In normal joints there was no significant difference with respect to response magnitude, latency and duration between the responses evoked by the purines (Figure 2). However, in arthritic joints, although there was no significant difference with respect to response magnitude and duration, the latency to onset of the slow ATP response was significantly longer than that of the adenosine response (Figure 2: ATP 2000 nmol 17.8 ± 2.4 s; adenosine 370 nmol 9.1 ± 2.0 s, $P<0.05$ Mann-Whitney).

In order to confirm that we were recording from afferents innervating the knee joint, we injected adenosine and ATP intra-articularly in three animals. Adenosine evoked a delayed increase in C-fibre afferent discharge when injected i.art., and ATP caused a biphasic increase (adenosine 370 nmol: 2.05 ± 0.59 impulses s^{-1}; ATP (slow component) 2000 nmol: 2.2 ± 0.54 impulses s^{-1}; PBS 0.1 ml: 0.01 ± 0.02 impulses s^{-1}; $n=2$ for each).

We compared the latencies to onset of the slow excitation evoked by the purines with those of the directly acting algogens, bradykinin and capsaicin. In C-fibres innervating normal knee joints, the onset latencies for the purines did not differ significantly from that for bradykinin. However, they were significantly ($P<0.05$) longer in comparison with the capsaicin response (adenosine 370 nmol: 8.8 ± 1.9 s $n=4$; ATP 2000 nmol: 9.3 ± 2.6 s $n=8$; bradykinin 9 nmol: 10.2 ± 2.5 s $n=12$; capsaicin 10 nmol: 1.1 ± 0.2 s $n=12$). The latency of the fast ATP response was similar to that of the capsaicin response (Dowd *et al*., 1998a).

We used selective purinoceptor agonists and antagonists to characterize the purinoceptor(s) mediating the delayed excitatory responses to adenosine and ATP. The adenosine A_1 receptor agonists, CPA (30 nmol i.a.) and GR79236 (85–285 nmol i.a.) also evoked a delayed increase in afferent discharge (CPA: see Figure 3; GR79236: 1.02 ± 0.17 impulses s^{-1}, $n=6$ afferents innervating normal joints). In contrast, the adenosine A_2 receptor agonist CGS21680 (190 nmol i.a.) had no effect on afferent discharge (normal joint: 0.01 ± 0.2 impulses s^{-1}, $n=2$; arthritic: 0.02 ± 0.1 impulses s^{-1}, $n=2$). The excitation evoked by adenosine and ATP was unaffected by the P2 receptor antagonist PPADS (16 μmol kg^{-1} i.a.), whereas, the adenosine A_1 receptor antagonist, DPCPX (3 μmol kg^{-1} i.a.) blocked the slow excitation associated with adenosine, ATP and CPA (Figure 3).

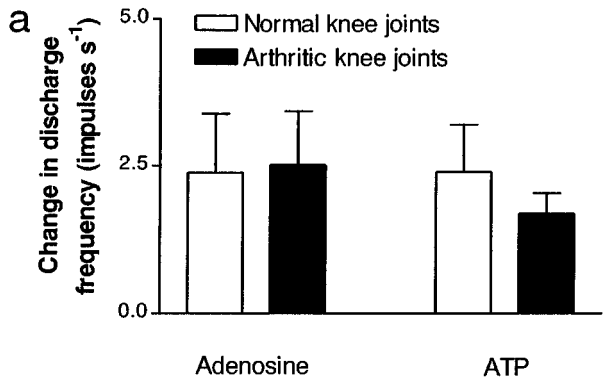
A significantly higher rate of spontaneous discharge was observed in C-fibre afferents innervating arthritic knee joints, in comparison with those recorded from normal joints (normal joints: 0.1 ± 0.01 impulses s^{-1}; arthritic: 0.39 ± 0.01 impulses s^{-1}, $P<0.05$). To determine whether endogenous adenosine contributed to this increase in basal activity, we studied the effect of DPCPX on the spontaneous discharge recorded from afferents innervating arthritic joints. DPCPX (3 μmol kg^{-1} i.a.) did not reduce the spontaneous discharge of adenosine-positive C-fibre afferents (spontaneous discharge before: 0.4 ± 0.2 impulses s^{-1}; after DPCPX: 0.3 ± 0.2 impulses s^{-1}, $n=6$).

Discussion

Our main finding is that adenosine excites the majority of C-fibre polymodal nociceptors innervating the rat knee joint. It also activated some Aδ-fibre afferents, but detailed study of these was not feasible because of their small number. The

excitation of C-fibres appears to be mediated *via* adenosine A_1 receptors because it was antagonized by the selective A_1 receptor antagonist DPCPX, and mimicked by the A_1 receptor agonists CPA and GR79236. The nucleotide ATP evoked a biphasic increase in afferent discharge consisting of a fast-onset response, mediated by P2X receptors (Dowd *et al*., 1998b), and a slow-onset response from rat knee joint nociceptors. In normal knees the slow component was similar in size, latency and duration to the adenosine-evoked response, and was antagonized by DPCPX. Thus, the delayed increase was likely to be mediated by adenosine, produced by metabolism of ATP, acting at metabotropic adenosine A_1 receptors.

The latency to onset of the responses evoked by adenosine did not differ significantly from that of a directly acting algogen, bradykinin, a finding compatible with adenosine also having a direct mode of action on the primary afferent terminals. Excitation of joint nociceptors by adenosine and ATP could be evoked by intra-articular injection of the purines, suggesting that the excitatory responses obtained were not secondary to effects on the vasculature. The evidence obtained makes it probable that the excitation of nociceptive afferents evoked by adenosine, either injected or produced by the metabolism of ATP, resulted from a direct action on nociceptive nerve terminals. It has recently been reported that adenosine causes a delayed excitation of vagal pulmonary C-fibre sensory terminals in rats through activation of A_1 receptors (Hong *et al*., 1998), which is consistent with our findings.



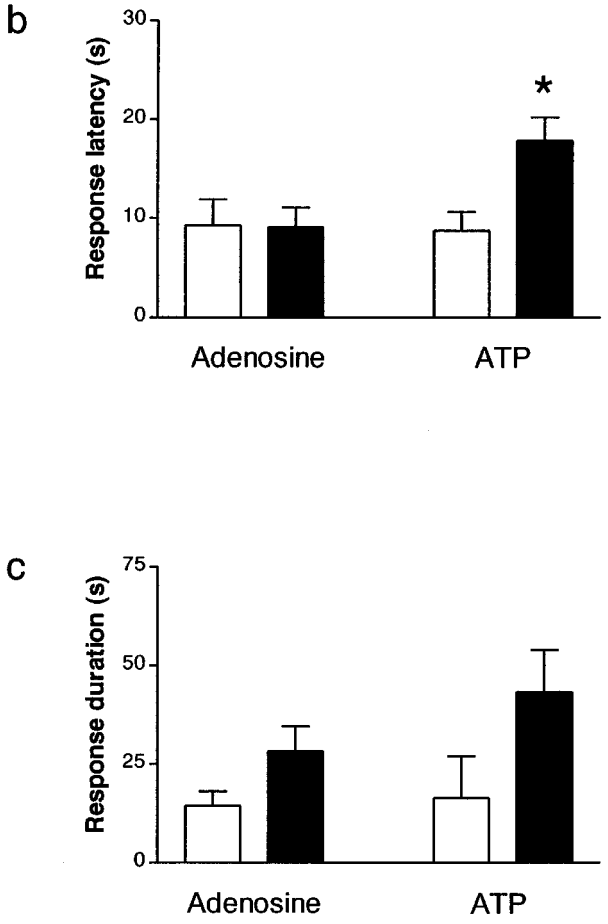


Figure 2 Summary of (a) amplitude (b) latency to onset and (c) duration of the response evoked in C-fibre afferents following i.a. injection of either adenosine (370 nmol) or ATP (2000 nmol). Data shown is from C-fibres innervating normal (adenosine: $n=8$; ATP $n=4$) or chronically arthritic (adenosine: $n=10$; ATP $n=13$) joints. Columns represent mean$\pm$s.e.mean. *$P<0.05$ ATP response latency in arthritic joints compared to (i) ATP response latency in normal joints and (ii) compared to adenosine response latency in arthritic joints.

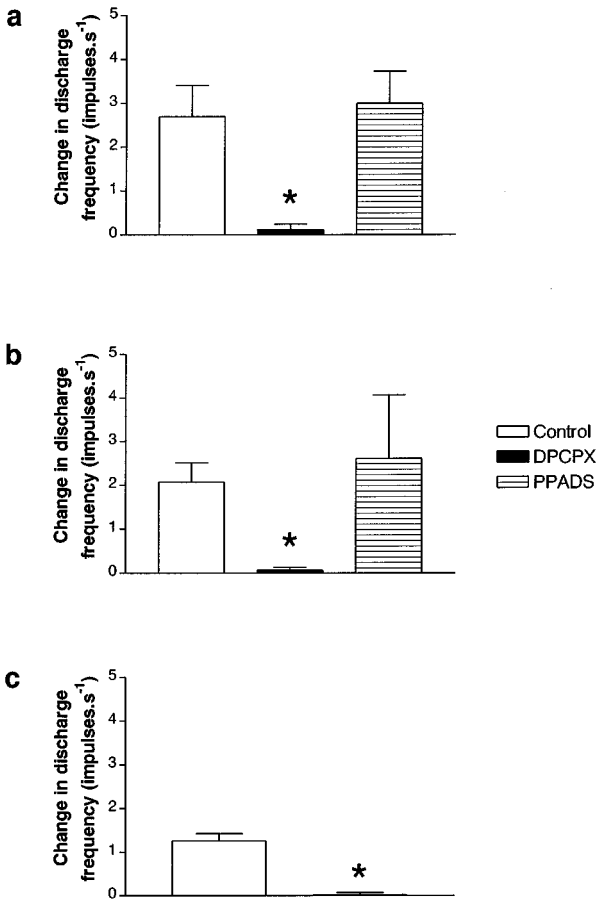


Figure 3 Slow-onset excitation evoked by intra-arterial injection of (a) adenosine (370 nmol), (b) ATP (2000 nmol) and (c) CPA (30 nmol) before and after either DPCPX (3 μmol kg^{-1}) or PPADS (16 μmol kg^{-1}). Pooled data from C-fibres innervating normal and arthritic knee joints (adenosine: control $n=16$, after DPCPX $n=4$, after PPADS $n=4$; ATP: control $n=12$, after DPCPX $n=3$, after PPADS $n=5$; CPA: control $n=8$, after DPCPX $n=5$). Columns represent mean$\pm$s.e.mean. *$P<0.05$ compared to control responses, Mann-Whitney test.

The magnitude and duration of the A_1-mediated afferent response to adenosine and ATP were unaffected by the presence of mild adjuvant-induced arthritis in the knee joint. However, the latency of the ATP-evoked slow response in arthritic knees was increased relative to its latency in normal joints, and also with respect to the latency for the response to adenosine in arthritic joints. This might be due to changes in the activity of ATP-metabolising enzymes in the arthritic knee joints because it is known that the half-life of ATP in the synovial fluid of patients with arthritis is increased (Park *et al*., 1996), that is, it is metabolized more slowly. This could explain why the latency to onset of the ATP response is increased in arthritic joints but the latency to onset of the adenosine response is not.

The finding that the adenosine A_1 receptor antagonist DPCPX did not reduce the elevated level of spontaneous activity observed in adenosine-sensitive nociceptive afferents recorded from chronically arthritic knee joints suggests that these afferents were not tonically activated by endogenous adenosine. However, this does not preclude a role for adenosine as a modulator of peripheral nociceptor sensitivity. The nucleoside may be released in a phasic manner, be enhancing the actions of another endogenous algogen, or influencing nociceptors over a longer time scale than the 3–4 hours that we were able to investigate in our experiments. Adenosine might also be important in the development of joint hyperalgesia, something our experiments on chronically inflamed joints did not address.

In contrast with this algogenic effect of peripheral adenosine A_1 receptor activation, activation of spinal adenosine A_1 receptors is associated with antinociception (see Sawynok, 1998 for review). Thus the overall role of A_1 receptors in nociception is likely to depend on the comparative release of adenosine in the spinal cord, the local concentration in the periphery and on the relative number of A_1 receptors. Because of these opposing effects, careful targeting of peripheral or spinal A_1 receptors will have to be considered if adenosine A_1 receptor antagonists or agonists are developed for the alleviation of pain.

In conclusion, both adenosine and ATP can excite the same nociceptive afferents in the rat knee joint, apparently by a direct action on A_1 receptors located on the peripheral nerve terminals. Local release of ATP during hypoxaemia and/or inflammation in joints could excite a population of nociceptive primary afferents, initially acting *via* P2X receptors then, following rapid metabolism of the nucleotide to adenosine, *via* activation of A_1 receptors. In addition, ATP and adenosine could also release algogens and enhance inflammation through actions on P1 and P2 receptors on nearby cells (e.g. synoviocytes, mast cells, blood vessels; Panayi, 1992; Scott *et al*., 1994). The role of adenosine and A_1 receptors associated with the peripheral terminals of nociceptive primary afferents innervating articular joints merits further study with a view to understanding their contribution to the development and maintenance of chronic pain associated with inflammatory joint disease.

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