The effects of inflammation and inflammatory mediators on nociceptive behaviour induced by ATP analogues in the rat

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1 We have studied the behavioural effects of intraplantar injections of adenosine 5'-triphosphate (ATP) and related compounds in freely moving rats and investigated whether these nociceptive effects are augmented in the presence of inflammatory mediators.

2 We find that in normal animals ATP and analogues produce dose-dependent nocifensive behaviour (seen as bursts of elevation of the treated hindpaw), and localized thermal hyperalgesia. The rank order of potency was: α,β -methyleneadenosine 5'-triphosphate (α,β -methylene ATP) > 2-methylthioadenosine triphosphate (2-methylthio ATP) > ATP. After neonatal treatment with capsaicin, to destroy small calibre primary sensory neurones, nocifensive behaviour was largely absent.

3 The effects of ATP analogues were assessed in three models of peripheral sensitization: 2 h after dilute intraplantar carrageenan (0.25% w v⁻¹); 24 h after irradiation of the hindpaw with ultraviolet (U.V.) B; immediately following prostaglandin E₂ (PGE₂) treatment. In all models the effect of α,β -methylene ATP was greatly augmented. After carrageenan, significant hindpaw-lifting behaviour activity was induced by injection of only 0.05 nmol of α,β -methylene ATP, some 100 times less than necessary in normal skin.

4 Our data suggest that it is much more likely that endogenous levels of ATP will reach levels capable of exciting nociceptors in inflamed versus normal skin. Our data also suggest the involvement of P2X₃ receptor subunits in ATP-induced nociception.

Keywords: Purinoreceptors; pain; nociceptor; sensitization; hyperalgesia

Abbreviations: α,β -methylene ATP, α,β -methyleneadenosine 5'-triphosphate; ADP, adenosine diphosphate; ATP, adenosine 5'-triphosphate; 2-methylthio ATP, 2-methylthioadenosine triphosphate; PBS, phosphate buffered saline; U.V.B., ultraviolet B

Introduction

ATP is well recognized as an energy source and modulator of cellular function, operating ubiquitously within the body. In the nervous system, ATP's role as a neurotransmitter has been established following studies demonstrating that ATP elicits fast excitatory potentials when applied to dorsal horn neurones (Jahr & Jessel, 1983; Fyffe & Perl, 1984; Salter & Henry, 1985) and dorsal root ganglion cells (e.g. Bean, 1990, see Brake & Julius, 1996 for review). In addition to its neurotransmitter role, there has been repeated speculation, dating back to the work of Holton (1959), that ATP might serve as mediator of pain. The discovery of a receptor for ATP, P2X₃, which is exclusively expressed by small diameter sensory neurones, presumed to be nociceptors (Chen *et al.*, 1995; Lewis *et al.*, 1995; Bradbury *et al.*, 1998), further suggests a nociceptive role for ATP. In a tissue culture system, Cook *et al.* (1997) reported that ATP analogues activated nociceptive neurones. The pharmacology of the responses generated was suggestive of P2X₃ receptor involvement.

Another compelling line of evidence for the role of ATP in pain relates to the algogenic effects of ATP in humans. ATP is reported to act consistently as a painful stimulus when applied to human blister bases (Bleehen & Keele, 1977), when injected intradermally, or when applied iontophoretically (unpublished

observations). The pain elicited is modest in magnitude and limited in duration to only a few minutes. This last feature may well result from the rapid biochemical degradation of ATP *in vivo*.

α,β -methylene ATP is a relatively stable ATP agonist due to its resistance to ATP-endonucleotidase degradation. It has been used in the only two studies to have examined pain-related behaviour induced by ATP in experimental animals. Bland-Ward & Humphrey (1997) gave rats intraplantar injections of ATP and related P2X agonists. They found that these agonists induced nocifensive behaviour—lifting and licking of the injected hindpaw. This behaviour, like the pain reported in human volunteers, persisted for a relatively brief period, measured in minutes and was dose-dependent. The same authors also reported that pre-treatment of the animals with bupivacaine or capsaicin abolished the nociceptive response, indicating that the lifting response was a consequence of hindpaw sensory nerve stimulation, in particular C or A δ fibres. The second behavioural study of the effects of ATP was performed by Sawynok & Reed (1997). These authors found that intraplantar injections of ATP facilitated the well-described nocifensive behaviour induced by intraplantar formalin injections. However, they did not see overt hindpaw-lifting or hindpaw-licking activity (greater than that seen with a saline injection), in response to either free ATP (50 or 500 nmol) or α,β -methylene ATP (50 nmol). Therefore, the first aim of the current work was to study the behavioural consequences of intraplantar ATP injection in an attempt to resolve these reports.

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A surprising feature of the published behavioural studies is that high doses of agonists have been required to induce effects—threshold doses for hindpaw-lifting behaviour were 100 nmol for α,β -methylene ATP and 1000 nmol for free ATP in the study by Bland-Ward & Humphrey (1997) and 50 nmol of ATP for potentiation of formalin-induced behaviour (Sawynok & Reid, 1997). It is not clear whether such high doses are likely to be realized by endogenous release of ATP, especially since the endogenous ligand, unlike some of the exogenously used analogues, is subject to rapid degradation. A second aim of the current work was to investigate whether ATP's effects might be enhanced under pathophysiological conditions. The rationale is that tissue injury is known to induce peripheral sensitization of nociceptors—that is, increase their sensitivity to a variety of stimuli including noxious heating, mechanical stimulation and several chemical agents such as bradykinin (Meyer *et al.*, 1994). We therefore tested the hypothesis that the nociceptive system might show an increased state of responsiveness to ATP and its analogues in the presence of experimental inflammation, and thus be active at low concentrations of ATP, such as those that might be present endogenously.

Methods

Animals used

Male Wistar rats weighing 225–325 g were housed in groups of five and maintained on a 12:12 h light/dark cycle at $22 \pm 2^\circ\text{C}$. Food and water were freely available. Each hindpaw of a particular animal was tested only once, and if both hindpaws were used, an interval of a week was left between successive tests. Animals tested with experimental inflammation models were used only once.

Responses to ATP analogues in normal animals

Groups of at least five normal rats were injected with a particular dose of a test ATP analogue. Prior to receiving an injection the animal was wrapped in cloth so as to expose only its hindpaw. Once calm, 100 μl of the test analogue was injected intradermally on the plantar surface of one hindpaw, using a 30G needle. One hundred μl of vehicle (phosphate buffered saline (PBS) pH 7.4) alone was injected as a control. Following the injection the animal was immediately placed in a Perspex box and a stop clock started. The hindpaw-lifting behaviour and responsiveness to noxious thermal stimuli were then assessed. The total time an animal spent with its hindpaw elevated clear of the box floor was measured in 2 min time bins following injection for a total of 6 min or 10 min in different experiments, depending whether thermal hyperalgesia was measured. Pilot experiments showed that more than 90% of hindpaw-lifting behaviour occurred within 6 min under all experimental conditions.

The hindpaw withdrawal time to noxious thermal stimulation of the plantar surface of the treated and untreated (contralateral) hindpaw was measured by the method of Hargreaves *et al.* (1988), using a commercial device (Model 7371, Ugo Basile, Italy). Baseline recordings were taken before and then 6 min following the injection. At both of these time points, three pairs of recordings were taken of the withdrawal latencies for each hindpaw, and then averaged.

Carrageenan inflammation

Fifty μl of 0.25% w v⁻¹ carrageenan was injected into the plantar surface of the rat's hindpaw. This dose is less than traditionally used to induce inflammation, but nonetheless produces a thermal hyperalgesia 2 h later (see Results). The sensitivity of the inflamed hindpaw to ATP analogues was assessed at this time in a similar fashion to that used for naïve animals (described above). The only difference was that the volume of ATP or vehicle solution injected was 50 μl (rather than 100:1), in order to minimize the nociceptive effects of distension of inflamed tissue.

PGE₂

The effects of the inflammatory mediator PGE₂ on the responses to ATP analogues were tested in other animals. PGE₂ (either 0.7 or 7 μmoles) was co-injected with α,β -methylene ATP into the plantar surface of one hindpaw (total volume of 100 μl). PGE₂ was also injected alone as a control. Hindpaw lifting behaviour was measured as before.

U.V.-irradiation

Hyperalgesia was induced by exposure of the left and right hindpaws to an ultraviolet B (U.V.B) light source (TL01, Phillips). Groups of five rats were anaesthetized using pentobarbitone at a dose 40 mg kg⁻¹. The plantar surface of hindpaws were then exposed to 1500 mJ cm⁻² of U.V.B with a wavelength of 311 nm. All other parts of the rat's body were protected from irradiation with aluminium foil and cloth. The 1500 mJ cm⁻² of U.V.B (equivalent approximately to twice the Minimal Erythemic Dose—MED) was delivered over approximately 14 min.

Six hours after the irradiation, the animals were tested with injections of ATP analogues (100 μl) into the one hindpaw, as above, and hindpaw-lifting time monitored for 6 min. This was repeated 24 h after the initial irradiation on the other irradiated hind hindpaw. Other animals were irradiated as above and tested with vehicle (PBS) injections.

Neonatal capsaicin treatment

In order to destroy the large majority of primary afferent nociceptors, litters of rats were given systemic injections of 50 mg kg⁻¹ capsaicin (10 g l⁻¹ solution dissolved in 10% v v⁻¹ ethyl alcohol, 10% v v⁻¹ tween 50, in saline) at 24 h and 72 h after birth (Nagy *et al.*, 1983). Groups of these rats were used 4 months later. They received the same treatments as the naïve animals. Female and male rats were used.

Compounds used

Adenosine 5'-triphosphate lithium salt and α,β -methylene ATP lithium salt were obtained from Sigma. 2-methylthioadenosine triphosphate tetrasodium was obtained from Tocris. All compounds were dissolved and diluted to the relevant molarity in phosphate buffered saline (PBS). The pH of each aliquot was checked and if found to be acidic (a feature of concentrated solutions) adjusted with a sodium hydroxide (40% w v⁻¹). Aliquots were stored at -70°C . On the day of use they were thawed and kept on ice prior to the injection. Prostaglandin E₂ (I.C.N.) was initially made up in ethanol (1 mg ml⁻¹) and subsequently diluted in PBS. Carrageenan (Sigma, type IV) was dissolved in saline at 0.25% w v⁻¹ and aliquots stored at -70°C until use.

Statistical analysis

The total hindpaw-lifting time exhibited by each animal was computed. Data are given here as group averages $\pm$ standard error, unless indicated otherwise. The responses of different groups of animals were compared by ANOVA followed by Dunnett or Dunn *post hoc* tests where appropriate. The Mann-Whitney Rank Sum Test was used to compare the lifting response in capsaicin treated animals with that observed in naïve animals. The withdrawal latencies before and after injection of PBS in naïve animals were compared using a paired *t*-test. The lifting response induced by α,β -methylene ATP alone was compared with that induced by co-administration of the same dose with PGE₂ using a *t*-test, as shown in Figure 6. A *t*-test was also carried out to compare the lifting response induced by α,β -methylene ATP plus PGE₂ with the hypothetical additive response calculated by adding the results of administering the compounds separately. A level of 5% was taken as evidence of statistical significance.

Results

Responses in normal rats

In normal rats, α,β -methylene ATP consistently induced periods of intermittent hindpaw-lifting behaviour which began within 10 or 20 s of injection. The animals also frequently orientated towards the injected hindpaw, and additionally showed episodes of licking and biting of the treated hindpaw. As illustrated in Figure 1, a 50 nmol dose of α,β -methylene ATP induced hindpaw-lifting behaviour that occupies about 50% of time for the first 4 min, but then subsides rapidly, with very little nocifensive behaviour present beyond 6 min. Injections of vehicle (PBS) into the hindpaw were associated with only a very limited amount of hindpaw-lifting and licking behaviour, Figure 1. For comparisons of different compounds and doses, the total hindpaw-lifting time exhibited by each animal in the 6 min after injection was calculated, Figure 2. The relative

Figure 1 The time spent (in 2 min time bins) in hindpaw-lifting in rats given an intraplantar injection of α,β -methylene ATP or PBS. The ATP was given to normal rats or rats treated as indicated in the legend. The pattern of nocifensive behaviour was the same in normal rats and in the presence of inflammatory mediators, with maximal hindpaw-lifting occurring within the first 5 min and rapidly diminishing thereafter. Negligible hindpaw-lifting was observed in rats injected with equal volume of phosphate-buffered saline vehicle. $n=5$ animals in all groups. Ten nmol of α,β -methylene ATP was given, except for carrageenan-treated rats, which received 5 nmol. Values are means $\pm$ s.e.mean.

potencies of free ATP, α,β -methylene ATP were investigated in normal animals. *In vivo*, ATP is quickly degraded by extracellular ectonucleotidases. α,β -methylene ATP is a specific P2X agonist and has the advantage of being less susceptible to degradation than ATP. 2-methylthio ATP is a non-specific P2-receptor agonist. Like ATP it is vulnerable to degradation by extracellular ectonucleotidases. All analogues elicited a dose related lifting response ($P<0.05$ in all cases, ANOVA, $n=5-10$ per group). α,β -methylene ATP was found to be more potent than free ATP or 2-methylthio ATP. A dose of 100 nmol of α,β -methylene ATP elicited twice as much lifting as 2-methylthio ATP, whilst the same dose of ATP elicited a negligible amount of lifting (Figure 2). One hundred μ l of the vehicle (PBS), injected alone, also induced minimal hindpaw-lifting (13.3 ± 6.4 s, $n=10$).

Hyperalgesia in normal animals

Using the method of Hargreaves, we observed a reduction in the withdrawal latencies to noxious thermal stimuli of hindpaws treated with ATP, α,β -methylene ATP or 2-methylthio ATP, as shown in Figure 3. However, only the changes produced by α,β -methylene ATP reached statistical significance ($P<0.01$, ANOVA, $n=5-10$ per group). Injection of vehicle produced no significant change in thermal threshold, tested in the same way ($P>0.2$, paired *t*-test, $n=10$).

Neonatal capsaicin treatment

To investigate the nature of the sensory neurones being stimulated by ATP and its analogues, experiments were carried out on rats treated neonatally with 1% w v⁻¹ capsaicin. Figure 4 shows that virtually all nociceptive behaviour was abolished following injections of free ATP (100 nmol) and α,β -methylene ATP (100 nmol and 10 nmol). Only in one group of animals (those tested with 1000 nmol of 2-methylthio ATP) was appreciable hindpaw-lifting behaviour still present, amounting to about 25% of that seen in normal animals. For all compounds the lifting response was significantly reduced in capsaicin treated animals compared to naïve animals ($P<0.02$ in each case, Mann-Whitney Rank Sum Test, $n=3-5$ per group).

Figure 2 Total hindpaw-lifting time occurring in the 6 min following intraplantar injections of different doses of free ATP, α,β -methylene ATP and 2-methylthio-ATP, injected in a volume of 100 μ l. $n=5-10$ for each drug/dose. Values are means $\pm$ s.e.mean. All compounds induce significant hindpaw lifting ($P<0.05$, ANOVA). The asterisks indicate which individual groups at a particular dose differ significantly from PBS (Dunnett's *post hoc* test).

Effects of ATP analogues in inflammatory models

Carrageenan inflammation A 50 μ l dose 50:1 of 0.25% carrageenan produced a modest thermal hyperalgesia of the treated paw 2 h later, as reflected by a 22% reduction in the withdrawal latency at this time ($P < 0.05$, paired t -test, comparing pre to post treatment). To reduce the nocifensive actions of tissue distension, only 50 μ l of test solutions were injected into carrageenan-inflamed rats. Figure 5 shows that hindpaw-lifting behaviour induced by α,β -methylene ATP was greatly augmented in carrageenan-inflamed skin ($P < 0.05$, two-way ANOVA, $n = 5$ per group). At a dose of 50 nmol, α,β -methylene ATP induced 311.6 ± 35.2 s ($n = 5$) of lifting compared to 81.8 ± 22.1 s ($n = 5$) in naïve. Perhaps more interestingly, significant hindpaw-lifting activity was induced by only 0.05 nmol of α,β -methylene ATP, some 100 times less than that necessary in normal skin. The time course of ATP-induced hindpaw-lifting was slightly prolonged in carrageenan-inflamed skin, Figure 1.

Figure 3 Thermal sensitivity of hindpaws determined 6 min after intraplantar injections of different doses of ATP, α,β -methylene ATP and 2-methylthio ATP, expressed as a percentage of the hindpaw withdrawal latency determined prior to intraplantar injection. Values less than 100 therefore indicate hyperalgesia. $n = 5-10$ for each drug/dose. Values are means $\pm$ s.e.mean. The effects of α,β -methylene ATP are statistically significant ($P < 0.05$, ANOVA).

Figure 4 Total hindpaw-lifting time in the 6 min following intraplantar injections of ATP, α,β -methylene ATP and 2-methylthio ATP, in naïve rats and in rats treated neonatally with capsaicin. $n = 3-5$ per drug per dose. The reduction in hindpaw-lifting time in capsaicin-treated rats is highly significant in all cases (single asterisk indicates $P < 0.05$; double asterisk indicates $P < 0.01$, Mann-Whitney Rank Sum t -test).

Co-administration with PGE₂ Pilot experiments were carried out to determine the effects of intraplantar injection of PGE₂ alone on hindpaw-lifting behaviour. Figure 6 shows that a dose of 7 μ moles induced 73.8 ± 17.3 s ($n = 5$) of hindpaw-lifting while 0.7 μ moles induced only 31.0 ± 10.2 s ($n = 5$). α,β -methylene ATP (1 nmol and 10 nmol) when co-injected with PGE₂ induced far greater nociceptive responses than in normal rats. Figure 6 shows the data for a dose of 1 nmol. At both doses of PGE₂, the lifting times were significantly greater than the additive lifting times induced by the two substances separately ($P < 0.05$, unpaired t -test, $n = 5$ per group). With

Figure 5 Nocifensive behaviour induced by intraplantar injections of α,β -methylene ATP or vehicle (50 μ l) into a hindpaw inflamed with carrageenan (50 μ l of 0.25%) 2 h previously. $n = 5$ per group. The total hindpaw-lifting time in the 10 min period following intraplantar injection is plotted. The increases seen in carrageenan-inflamed skin are significant ($P < 0.05$, ANOVA). Asterisks indicate significant differences ($P < 0.05$) between inflamed and normal animals (Dunnett's *post hoc* test).

Figure 6 Effect of PGE₂ on lifting behaviour induced by 1 nmol of α,β -methylene ATP. PGE₂ was given at a dose of 0.7 or 7 μ mol either alone or mixed with 1 nmol of α,β -methylene ATP, and the ensuing hindpaw-lifting time accumulated over the following 10 min $n = 5$ for each group. PGE₂ potentiates ATP-induced hindpaw-lifting behaviour. Asterisks indicate a significant difference between groups (single asterisk $P < 0.05$, double asterisk $P < 0.01$, Mann-Whitney Rank Sum t -test).

Figure 7 Effect of U.V. inflammation on hindpaw-lifting behaviour occurring in the 6 min after intraplantar injections of 1000 nmol of ATP, 100 nmol of α,β -methylene ATP or vehicle. The dose of U.V.B irradiation given was 1500 mJ cm^{-2} . U.V. inflammation potentiates the nocifensive activity of ATP analogues. The paw lifting behaviour was significantly augmented for both compounds ($P < 0.05$, ANOVA). The asterisks indicate significant difference between individual groups at a particular dose ($P < 0.05$, Dunnett's *post hoc* test).

0.7 μmoles of PGE_2 , α,β -methylene ATP (10 nmol) induced $134.6 \pm 29.5 \text{ s}$, ($n=5$) of hindpaw-lifting compared to a calculated additive response of 84.9 s. With a higher dose of PGE_2 , 7 μmoles α,β -methylene ATP (10 nmol) induced almost double the projected lifting response. Thus, the effect of α,β -methylene ATP is potentiated in the presence of PGE_2 .

U.V. irradiation Initial experiments showed that the dose of U.V.B used produced a thermal hyperalgesia of the treated skin. Withdrawal latencies to noxious thermal stimuli decreased from $10.2 \pm 1.5 \text{ s}$ to $9.2 \pm 0.7 \text{ s}$ at 6 h and $8.5 \pm 0.9 \text{ s}$ at 24 h. The change at 24 h was statistically significant ($P < 0.05$ *t*-test, $n=5$ per group). Animals were tested 6 and 24 h following irradiation with α,β -methylene ATP (100 nmol) and free ATP (1000 nmol). The total hindpaw-lifting behaviour was significantly augmented in the presence of U.V. inflammation for both compounds ($P < 0.05$, ANOVA, $n=5$ per group), Figure 7. The hindpaw-lifting behaviour to vehicle was not significantly ($P > 0.06$, ANOVA $n=8$ per group) increased in U.V. inflamed skin.

Discussion

Behavioural responses in naïve animals

The behavioural responses induced by the intraplantar injections of ATP and its related agonists α,β -methylene ATP and 2-methylthio ATP strongly suggest these compounds elicit a brief period of pain or local irritation. The lack of such responses in animals treated neonatally with capsaicin also indicates that ATP responses were pain-related, since this treatment selectively destroys small calibre sensory nerve fibres, more than 90% of which are known to be nociceptors (see Snider & McMahon, 1998). The very limited behavioural response remaining in some of the animals probably reflects the fact that destruction of nociceptors is usually incomplete—typically 10% or more fibres survive (Nagy *et al.*, 1983). Local (primary) thermal hyperalgesia was also apparent with some stimuli—those producing the greatest nocifensive behaviour—suggesting that ATP provokes thermal sensitization of

nociceptive terminals in skin. Similar sensitization is seen with other chemical algogens such as bradykinin (see Meyer *et al.*, 1994).

All three compounds induced hindpaw-lifting behaviour with the same time course, suggesting a common mechanism of action. The dose-dependence of the nociceptive response is consistent with a receptor-mediated event. Only the ipsilateral hindpaw responded to the injections; the lack of response in the contralateral hindpaw implies a peripherally mediated mechanism. In normal animals, our findings are very similar to those reported by Bland-Ward & Humphrey (1997), in terms of the qualitative nature of responses, including time-course, dose-dependence, and the relative potencies of α,β -methylene ATP versus free ATP. While Sawynok & Reid (1997) reported pro-nociceptive effects of ATP and α,β -methylene ATP (potentiation of formalin second phase response), they did not see any overt nocifensive behaviour attributable to either of these agonists, at doses we here find effective. It is not clear what methodological or other differences might account for the different findings. One salient feature, however, might be that ATP-induced pain in humans is quite modest in intensity (unpublished observations). This, coupled with the short duration of effect of ATP, might render the behavioural responses susceptible to stress-induced analgesia associated, for example, with handling and injection.

Behavioural responses in models of peripheral sensitization

We report here that nociceptive actions of ATP are markedly augmented in the presence of inflammation or inflammatory mediators. We saw both an increase in nocifensive behaviour to ATP analogues and a striking reduction in threshold doses. The most obvious functional implication of this result is that it is much more likely that extracellular levels of ATP will reach levels capable of activating nociceptors in inflamed tissues. Doses of only 0.05 nmol were effective in the pathophysiological states, approximately 100 times less than in normal tissue. It is interesting that the two human psychophysical studies in the literature also show different threshold doses at which ATP produces pain: when injected in normal skin, Coutts *et al.* (1981) found that doses of about 250 nmol of ATP were necessary to produce pain, but on blister bases pain was reported with doses of 0.2 to 0.6 nmol (calculated from data in Bleehen & Keele, 1977). It may be that the injury associated with the creation of a blister base is sufficient to sensitize nociceptors.

There are several ways in which the enhanced responses seen here might arise. One possibility is that changes in the pH of the extracellular environment might account for the augmented behaviour, since there is ample evidence that pH can modify responses of P2X receptors when studied in culture (Wildman *et al.*, 1997; Li *et al.*, 1997). Inflammation can also be associated with tissue acidosis (see Steen *et al.*, 1992). However, this cannot account for the changes seen with PGE_2 because these occurred in normal skin. Whilst carrageenan and U.V. treated skin might become acidic, the ATP analogues used in our studies were delivered in buffered vehicle. One hallmark of inflamed tissue is, of course, increased tenderness to tactile stimuli. However, the augmentation seen here cannot be due to this factor, because we controlled for the effects of volume *per se* using vehicle. Changes in CNS excitability (so-called central sensitization—McMahon *et al.*, 1993) are not features of PGE_2 or U.V. inflammation, and are therefore unlikely contributors to changes seen here.

Another possibility is that inflammatory stimuli might up-regulate receptor levels in nociceptors—for instance as is reported for bradykinin receptors (Petersen *et al.*, 1996). While this might be a feasible explanation for the altered responses seen after U.V. irradiation, the effects seen with carrageenan and PGE₂ are too rapid to reflect altered gene expression. It is more likely that some modification of the receptor, such as its state of phosphorylation, could modify its sensitivity, perhaps by altering the rate at which it desensitizes. Calcium-dependent dephosphorylation appears to be a common mechanism for desensitization of various ligand-gated ion channels (see Yakel, 1997).

The inflammatory stimuli used here are known to activate second messenger systems within primary afferent nociceptors (Pitchford & Levine, 1991) and produce a peripherally mediated hyperalgesia (Moncada *et al.*, 1975; Hargreaves *et al.*, 1988; Urban *et al.*, 1993).

Nature of receptors mediating nociceptive responses to ATP

Circumstantial evidence suggests that the nociceptive effects of ATP are likely to involve P2X₃ receptors, for the following reasons: (i) α,β -methylene ATP, the most potent ligand in the present study, is known to be relatively selective for P2X₁ and P2X₃ receptors (Rongen *et al.*, 1997; Fredholm *et al.*, 1997), and little P2X₁ mRNA is found in dorsal root ganglia (Lewis *et al.*, 1995; Kidd *et al.*, 1995; Collo *et al.*, 1996); P2X₃ is selectively expressed in small neurones, known to be

predominantly nociceptive in nature (Chen *et al.*, 1995; Lewis *et al.*, 1995); (ii) Artificial assembly of P2X₃ and P2X₂ subunits results in a channel with a unique phenotype which produces a current resembling that induced by ATP in the native channel expressed by nociceptors (Cook *et al.*, 1997). (iii) Adenosine and ADP, the main degradation products of ATP, do not elicit substantial hindpaw-lifting following intraplantar injections into the rat hindpaw (Bland-Ward & Humphrey, 1997), making it unlikely that P1 or P2Y receptor activation contributes to the responses seen.

The lack of specific tools (e.g. selective agonists or antagonists of different receptors subtypes, knock-out animals etc.) make a more precise assessment difficult.

Together our results provide further evidence that ATP may function as a peripheral mediator of pain. In particular, it may be important in inflammatory or other pathophysiological conditions, since at concentrations that are likely to be physiologically relevant, ATP generates a nociceptive response only in the presence of inflammatory mediators. This latter finding may also explain why some workers have failed to see effects of ATP in intact dorsal root ganglion cells (Stebbing *et al.*, 1998), where the conditions used may have been associated with sub-optimal receptor sensitivity.

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