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Acute nociception mediated by hindpaw P2X receptor activation in the rat

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1 The functional consequences of P2X receptor activation on peripheral sensory neurones have been investigated *in vivo*. Behavioural indices of acute nociception were monitored in the conscious rat following subplantar injection of adenosine 5′-triphosphate (ATP), α,β-methylene ATP, adenosine 5′-diphosphate (ADP) and adenosine.

2 Signs of overt nociception, i.e. hindpaw lifting and licking, were apparent in animals injected subplantar with the P2X receptor agonist, α,β-methylene ATP. Nociceptive behaviours continued for 15 min following administration of α,β-methylene ATP (200 nmol) and were dose-related (0–5 min hindpaw lifting times after injection of α,β-methylene ATP 100 nmol and 1000 nmol were 89±26 s and 232±11 s, respectively). Subplantar ATP evoked a modest response only at the highest dose tested (1000 nmol; 0–5 min hindpaw lifting time 66±19 s) whilst ADP or adenosine (both 600 nmol) elicited negligible spontaneous nociceptive activity.

3 Morphine (3 mg kg^{-1}, i.v.) abolished hindpaw licking behaviour induced by subplantar injection of either α,β-methylene ATP (600 nmol) or bradykinin (1 nmol) and substantially reduced (88±5%) paw licking in formalin (0.5%, 0.1 ml) injected animals. In contrast, hindpaw lifting was only modestly inhibited (34±11%) in morphine-pretreated animals that had received subplantar bradykinin and was unaffected in rats in which the noxious stimulus was either subplantar α,β-methylene ATP or formalin. Pretreatment of hindpaws with subplantar bupivacaine (1% w/v, 0.1 ml) abolished α,β-methylene ATP-evoked nociceptive behaviours.

4 Hindpaw lifting and licking mediated by α,β-methylene ATP (600 nmol, subplantar) were inhibited (72±15% and 95±5%, respectively) by 30 min local pretreatment with 600 nmol α,β-methylene ATP. Subplantar α,β-methylene ATP pretreatment did not inhibit behaviour stimulated by subsequent bradykinin (1 nmol) or formalin (0.5%, 0.1 ml) injection into the hindpaw.

5 Desensitization of small diameter sensory neurones with a single subplantar injection of capsaicin (100 μg) abolished all behaviours indicative of spontaneous nociceptive sensation in animals subsequently injected with α,β-methylene ATP (600 nmol), bradykinin (1 nmol) or formalin (0.5%, 0.1 ml).

6 We conclude that activation of P2X receptors present on small diameter (capsaicin-sensitive) primary afferent neurones in the rat hindpaw mediates behaviour indicative of acute nociception.

Keywords: P2X purinoceptors; α,β-methylene ATP; spontaneous nociception; sensory neurones; hindpaw lifting; subplantar

Introduction

Adenosine 5′-triphosphate (ATP) functions as an intercellular messenger by interacting with membrane receptors (P2 purinoceptors) present on a broad range of cell types. ATP receptors can be divided, on the basis of structure, into two receptor classes: G-protein coupled receptors (i.e. P2Y) and the ATP-gated cation channels (i.e. P2X receptors) (for recent reviews see Burnstock, 1996 and North, 1996). It is apparent that functional P2X receptors are composed of a number of discrete subunits, each encoded by a different gene. To date, the cDNAs relating to seven separate P2X receptor subunits (designated $P2X_{1-7}$) have been cloned from a variety of sources (Valera *et al*., 1994; Brake *et al*., 1994; Chen *et al*., 1995; Lewis *et al*., 1995; Collo *et al*., 1996; Buell *et al*., 1996; Surprenant *et al*., 1996).

P2X receptor subunits, when expressed in cultured mammalian cells or *Xenopus* oocytes, are presumed to combine with unknown stoichiometry to form homomultimeric ATP-gated cation channels. The functional characteristics of recombinant P2X receptors differ markedly. For example, $P2X_4$ and $P2X_6$ receptors are insensitive to antagonists such as suramin and pyridoxal-5-phosphate-6-azophenyl-2′4′-disulphonic acid (PPADS) (Buell *et al*., 1996; Wang *et al*., 1996). Furthermore, $P2X_1$ and $P2X_3$ receptors are sensitive to activation by α,β-methylene ATP and desensitize rapidly (Valera *et al*., 1994; Chen *et al*., 1995; Lewis *et al*., 1995), whilst in contrast the $P2X_2$ and $P2X_4$ subtypes are unresponsive to α,β-methylene ATP and do not desensitize in the continuous presence of ATP (Brake *et al*., 1994; Evans *et al*., 1995; Buell *et al*., 1996; Wang *et al*., 1996). Heteropolymerisation studies indicate that certain features of each P2X subunit can confer specific functional qualities to the finally assembled receptor. For example, co-expression of $P2X_2$ and $P2X_3$ subunits in oocytes yields a P2X receptor which is non-desensitizing and α,β-methylene ATP sensitive. Thus, as with other ligand-gated ion channels (e.g. nicotinic and NMDA receptors), heteropolymerisation of P2X subunits *in vivo* could yield a diverse range of functionally distinct ATP-gated receptors.

The distribution of P2X receptors in the rat nervous system has been deduced by *in situ* hybridization techniques (Kidd *et al*., 1995; Collo *et al*., 1996). These studies have implied the presence of P2X receptors (principally $P2X_4$ and $P2X_6$) within the dorsal horn of the spinal cord, particularly in the substantia gelatinosa where sensory neurones synapse. This suggests that ATP released from primary afferent nerves may participate in the central processing of sensory information. In this respect, studies highlighting the localization of P2X receptors in the dorsal horn support previously obtained observations that certain dorsal horn neurones depolarize rapidly

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on exposure to ATP both *in vitro* (Jahr & Jessell, 1983) and *in vivo* (Fyffe & Perl, 1984; Salter & Henry, 1985).

Recently, interest in the role of ATP in sensory function has been renewed by the demonstration that $P2X_3$ mRNA displays a restricted distribution in the rat, being localized exclusively in small diameter sensory neurones of the dorsal root, nodose and trigeminal ganglia (Chen *et al*., 1995; Lewis *et al*., 1995). This finding has prompted the hypothesis that ATP liberated from damaged tissue may stimulate nociceptors to initiate pain sensation. The $P2X_3$ receptor has thus been proposed as a possible target for pharmacological intervention in the treatment of pain in man (Kennedy & Leff, 1995; Burnstock, 1996; Burnstock & Wood, 1996). However, whilst it has been demonstrated by means of electrophysiological techniques *in vitro* that ATP can (i) gate inward currents through $P2X_3$ receptors in dissociated rat dorsal root ganglion neurones (Bean, 1990; Robertson *et al*., 1996) and (ii) activate cutaneous afferent neurones in a neonatal rat tail-spinal cord preparation (Trezise & Humphrey, 1997), to date there has been no clear demonstration that ATP elicits pain perception in conscious animals resulting from P2X receptor activation. Therefore, the aim of present study was to examine the behavioural effects of subplantar injection of either ATP or its hydrolysis-resistant analogue, α,β-methylene ATP, in the conscious rat.

Methods

Animals

Male Random Hooded rats (150–250 g; Glaxo Wellcome) were obtained at least one week before use and were housed, not more than 5 to a cage, in a purpose-built holding facility providing a 12 h light/dark cycle (lights on 07 h 00 min) maintained at constant temperature ($21\pm2^{\circ}$C). All experiments were carried out between 11 h 00 min and 18 h 00 min. Animals were transported to the behavioural testing laboratory at least 1 h before the start of each experiment and were allowed free access to food and water until the time of subplantar algogen administration. Each animal was used only once for behavioural or hindpaw volume experiments and was humanely killed at the end of each experiment. The research complied with national legislation and with Glaxo Wellcome Policy on the Care and Use of Animals and with related codes of practice.

The effect of subplantar injection with purinoceptor agonists, bradykinin or formalin

In behavioural experiments, animals were lightly restrained and given a single subplantar injection into the left hindpaw, by use of a 26 G needle, of test algogen before immediate transfer to a clear perspex observation chamber (base 20×30 cm, height 20 cm). A mirror placed behind the observation chamber enabled the observer to view the injected hindpaw at all times. Rats were then observed continuously and the duration of time engaged in either hindpaw lifting or hindpaw licking activity was timed separately by digital stopwatches. Two preliminary experiments were carried out for the purpose of assay optimization. First, animals injected with either α,β-methylene ATP (200 nmol, subplantar), bradykinin (10 nmol, subplantar) or formalin (0.5%, 0.1 ml subplantar) were observed for both hindpaw lifting and licking activity over the 30 min period following algogen injection. These activities were expressed as duration (s) per 5 min epoch and the total area under the curve (AUC; min^2) for the entire observation period. Secondly, animals were injected subplantar with one of a range of doses of either α,β-methylene ATP (100–1000 nmol), bradykinin (0.1–10 nmol), ATP (100–1000 nmol), adenosine 5′-diphosphate (ADP; 600 nmol) or adenosine (600 nmol) and observed for hindpaw lifting only, during the 5 min period following injection.

Subsequently, animals were observed for both hindpaw lifting and licking activity over the 5 min period immediately following subplantar injection of either 600 nmol α,β-methylene ATP, 1 nmol bradykinin or formalin (0.5%, 0.1 ml). Results are expressed as time engaged in either hindpaw lifting or licking activity during the 5 min observation period. In some experiments animals were pretreated with either morphine (3 mg kg^{-1}, i.v.), bupivacaine (1% w/v, 0.1 ml, subplantar) or α,β-methylene ATP (600 nmol, subplantar) before subplantar administration of the algogenic substance under study. In order to investigate the nature of the nerve fibres conveying sensory information after subplantar injection of either α,β-methylene ATP, formalin or bradykinin, some animals were pretreated with capsaicin (100 μg, subplantar) under brief general anaesthesia (5 min; isofluorane 3% in $N_2O:O_2$) 3 h before behavioural testing.

Hindpaw volume measurements were made with a hindpaw plethysmometer (Ugo Basile, Italy). Left hindpaw volume measurements were made before and at 5 min and 30 min after single subplantar injection of either α,β-methylene ATP (200 or 600 nmol), bradykinin (1 or 10 nmol) or phosphate buffered saline (PBS; 0.1 ml).

Compounds and solutions

Adenosine 5′-triphosphate disodium salt (ATP), adenosine 5′-diphosphate sodium salt (ADP), α,β-methylene ATP lithium salt, adenosine, bradykinin acetate, 8-methyl-N-vanillyl-6-nonenamide (capsaicin) and bupivacaine hydrochloride were all obtained from Sigma. Morphine HCl was purchased from AAH Pharmaceuticals Ltd. (U.K.). All algogens were dissolved and diluted to the appropriate concentration in freshly prepared phosphate-buffered saline (PBS; composition in mM: NaCl 138, KCl 2.7, KH_2PO_4 1.5, Na_2HPO_4 4.3; pH 7.4) and kept on ice until subplantar administration. In experiments where animals were pretreated with subplantar injections of bupivacaine or α,β-methylene ATP these solutions were prepared in 0.9% w/v sodium chloride. The vehicle for capsaicin was ethanol (10% v/v) and Tween 80 (10% v/v) in 0.9% w/v sodium chloride.

Statistical analysis

Data are expressed as mean$\pm$s.e.mean. Differences between treatment groups were assessed by Student's t test or, where appropriate, ANOVA followed by Dunnett's *post-hoc* test for multiple comparisons. In all cases a probability (P) value of <0.05 was considered to indicate statistical significance.

Results

Subplantar administration of ATP analogues and bradykinin

In preliminary experiments, rats responded to a single subplantar injection of the ATP analogue, α,β-methylene ATP (200 nmol), by guarding, lifting, licking and biting the affected hindpaw. These typically nociceptive behaviours were most intense during the first 5 min following α,β-methylene ATP injection but had completely disappeared 20 min later. Hindpaw lifting (AUC 23.5 ± 3.5 min^2, $n=8$) was the predominant behaviour observed and was considerably greater than hindpaw licking at all time points studied (AUC 3.0 ± 0.8 min^2, $n=8$; $P<0.001$ when compared with hindpaw lifting) (Figure 1). Subplantar injection of bradykinin (10 nmol) evoked a qualitatively similar pattern of behaviour to that observed in α,β-methylene ATP-injected animals. Thus, animals displayed 10 min of marked hindpaw lifting activity after bradykinin injection, whilst paw licking was, again, less pronounced (AUC for bradykinin-evoked hindpaw lifting and hindpaw licking activity were 19.8 ± 2.4 min^2 and 2.4 ± 0.5 min^2, respectively, $n=5$ for both; $P<0.001$) (Figure

1). There was no evidence of the development of a late-phase response after either α,β-methylene ATP or bradykinin injection as seen following subplantar formalin injection. Phosphate-buffered saline (PBS), the vehicle for both α,β-methylene ATP and bradykinin, failed to elicit responses indicative of spontaneous nociception in all animals tested. Thus, it was noted that the minimal hindpaw lifting observed up to 30 min after subplantar PBS injection (30 ± 24 s, $n=4$) was attributable to normal rearing and grooming behaviour, whilst no licking activity was apparent.

Separate experiments were carried out in order to establish whether hindpaw oedema formation is an important factor in the development of the spontaneous nociceptive behaviours evoked by subplantar administration of α,β-methylene ATP or bradykinin. No significant difference ($P>0.05$) was observed between the volume of hindpaws injected with 200 nmol α,β-methylene ATP and control, PBS-injected, hindpaws at either 5 min or 30 min after subplantar injection. In contrast, subplantar injection of 10 nmol bradykinin resulted in a rapidly developing inflammation of the treated hindpaws. Thus, 5 min after bradykinin administration mean hindpaw volume had increased by about 40% ($P<0.05$ cf control, $n=5$) which was maintained 30 min later (Figure 2). There was no significant difference between the pretreatment hindpaw volumes of animals assigned to either control, α,β-methylene ATP or bradykinin treatment groups (i.e. left hindpaw volumes 1.09 ± 0.07 ml, 1.11 ± 0.06 ml and 1.10 ± 0.04 ml respectively, all $n=5$; $P>0.05$).

Experiments conducted in order to investigate the relative potencies of α,β-methylene ATP and bradykinin revealed that hindpaw lifting responses were clearly dose-related (Figure 3). Bradykinin was considerably more potent than α,β-methylene ATP in this respect; for example, calculated doses of bradykinin and α,β-methylene ATP required to produce a notional hindpaw lifting time of 150 s over the 5 min observation period were approx. 4 nmol and 300 nmol, respectively. In contrast, subplantar ATP was a poor stimulant of hindpaw lifting, producing only a modest hindpaw lifting response (0–5 min hindpaw lifting time, 66 ± 19, $n=6$) at a dose of 1000 nmol, whilst at a lower dose (200 nmol) ATP was without effect (Figure 3).

In a separate experiment, the P1 purinoceptor agonist, adenosine (600 nmol), and the preferential P2Y receptor agonist, ADP (600 nmol), elicited negligible hindpaw lifting activity in the 5 min period following subplantar injection (i.e. 0–5 min hindpaw lifting times for adenosine and ADP were

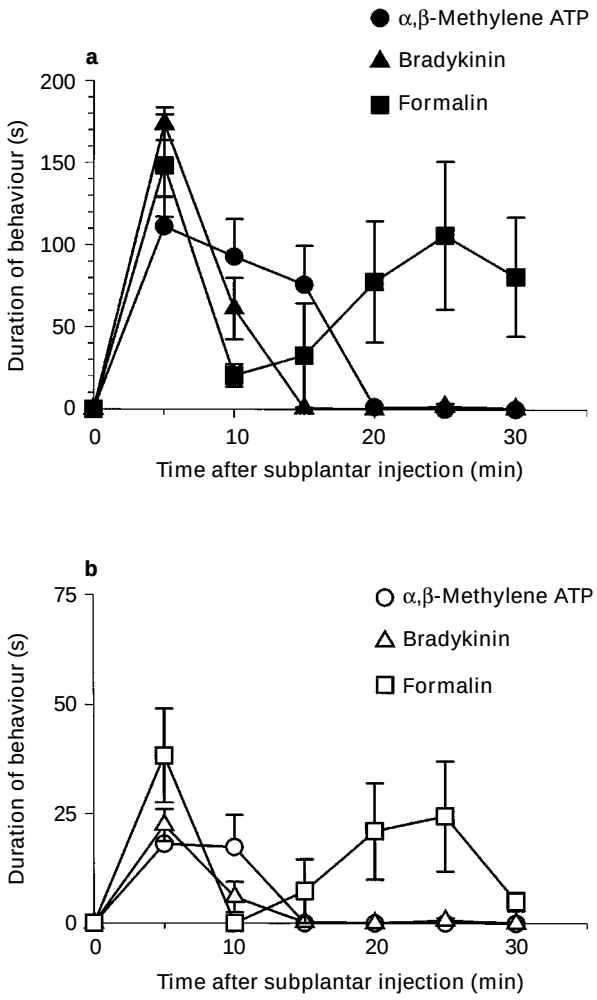

Figure 1 Time course of hindpaw lifting (a) and hindpaw licking (b) behaviours in rats injected subplantar with either α,β-methylene ATP (200 nmol), bradykinin (10 nmol) or formalin (0.5%, 0.1 ml). Note the difference in scales between panels. Negligible hindpaw lifting and licking activity was observed in animals injected with an equal volume of phosphate-buffered saline vehicle. Data points indicate mean and vertical lines show s.e.mean ($n=5-8$ animals).

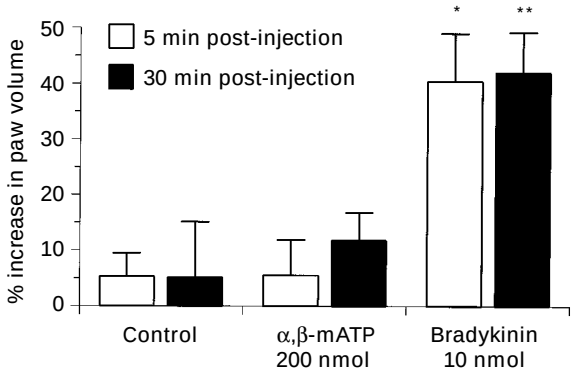

Figure 2 Increase in volume of left hindpaw in rats 5 min and 30 min after subplantar administration of phosphate-buffered saline vehicle (0.1 ml; Control), α,β-methylene ATP (200 nmol) or bradykinin (10 nmol). Left hindpaw volumes before subplantar administration were 1.09 ± 0.07 ml, 1.11 ± 0.06 ml and 1.10 ± 0.04 ml for phosphate buffered saline, α,β-methylene ATP and bradykinin treatment groups, respectively. Columns represent mean ± s.e.mean ($n=5$ animals in each group). $^*P<0.05$ compared with control at 5 min, $^{**}P<0.05$ compared with control at 30 min. Similar results were also obtained 5 min after subplantar injection of α,β-methylene ATP (600 nmol) or bradykinin (1 nmol) in separate experiments (see text).

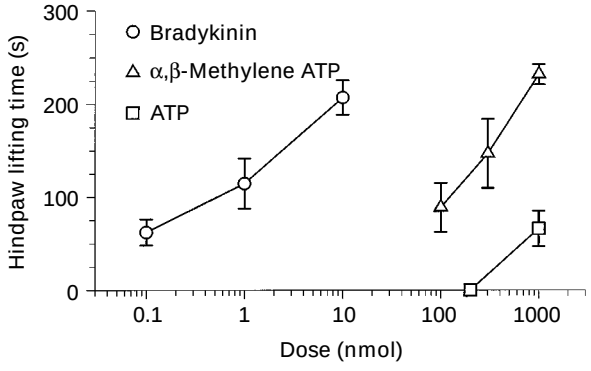

Figure 3 The effect of varying subplantar doses of bradykinin, α,β-methylene ATP or ATP on hindpaw lifting behaviour in rats. Hindpaw lifting times were determined during the 5 min period immediately following subplantar algogen injection. No hindpaw lifting behaviour was observed in control animals injected subplantar with phosphate buffered saline ($n=4$ animals). Plotted data points indicate mean and vertical lines show s.e.mean ($n=5-6$ animals in each group).

13.5±8.3 s and 7.3±2.7 s, respectively, $n=6$ for both), whilst subplantar administration of α,β-methylene ATP (600 nmol) resulted in hindpaw lifting behaviour totalling 217.2±7.9 s during the same period ($n=6$).

In all subsequent experiments α,β-methylene ATP and bradykinin were employed at subplantar doses of 600 nmol and 1 nmol, respectively, and nociceptive responses were monitored only for the 5 min immediately after hindpaw injection. It was therefore considered necessary to investigate whether hindpaw swelling develops as a consequence of subplantar injection of either α,β-methylene ATP (600 nmol) or bradykinin (1 nmol) during this period. Hindpaw volume was not significantly increased in animals 5 min after subplantar injection of 600 nmol α,β-methylene ATP (9.8±3.2% increase in left hindpaw volume, $n=6$) when compared with PBS-treated control animals (5.9±2.2% increase in hindpaw volume, $n=6$; $P>0.05$). In contrast, a significant increase in hindpaw volume was observed 5 min after subplantar administration of 1 nmol bradykinin (31.4±2.4% increase in hindpaw volume, $n=6$; $P<0.05$ when compared with control group).

The effect of systemic morphine or local bupivacaine pretreatment on α,β-methylene ATP-evoked behaviours

Nociceptive responses observed in the 5 min period after subplantar injection of α,β-methylene ATP (600 nmol), bradykinin (1 nmol) or formalin (0.5%, 0.1 ml) were compared in animals that had been pretreated with either 0.9% w/v sodium chloride (2 ml kg^{-1}, i.v.; control) or morphine (3 mg kg^{-1}, i.v.) 10 min before algogen administration. Control animals responded similarly to all three agents (Figure 4). Morphine abolished hindpaw licking in animals that had been injected subplantar with either α,β-methylene ATP or bradykinin and greatly inhibited (i.e. 88±5%) formalin-induced hindpaw licking (all $P<0.01$, $n=8$). In contrast, hindpaw lifting behaviour was less susceptible to inhibition by morphine with only a modest (i.e. 34±11%; $P<0.05$, $n=8$) inhibition of hindpaw lifting seen in animals that had received subplantar bradykinin. Hindpaw lifting evoked by either α,β-methylene ATP or formalin was not significantly inhibited ($P>0.05$) by morphine pretreatment (Figure 4).

In a separate experiment, 30 min pretreatment with the local anaesthetic bupivacaine (0.1 ml, 1% w/v, subplantar) significantly inhibited hindpaw lifting and licking behaviours elicited by a subsequent subplantar injection of 600 nmol α,β-methylene ATP. Thus, paw lifting times in the 5 min period after subplantar α,β-methylene ATP injection in bupivacaine and control, 0.9% w/v saline, pretreated animals were 4.0±2.6 s and 134.2±15.7 s, respectively ($P<0.01$, $n=6$ for both groups). In the same animals, hindpaw licking responses were reduced from 22.5±10.3 s (control, $n=6$) to 1.7±1.2 s after local bupivacaine ($P<0.01$, $n=6$).

Desensitization of α,β-methylene ATP-evoked behavioural responses

Additional experiments were carried out to investigate whether α,β-methylene ATP-evoked behavioural responses diminish upon continued exposure to this stable ATP analogue. Animals were pretreated with a subplantar injection of either α,β-methylene ATP (600 nmol) or vehicle (PBS, 0.1 ml) 30 min before a further subplantar injection of either α,β-methylene ATP (600 mol), bradykinin (1 nmol) or formalin (0.5%, 0.1 ml). Nociceptive responses evoked by α,β-methylene ATP were considerably reduced in rats that had previously received α,β-methylene ATP, when compared with vehicle-treated animals (i.e. hindpaw lifting and licking were inhibited by 72±15% and 95±5%, respectively; $P<0.01$, $n=6$ for both) (Figure 5). In contrast, 30 min subplantar pretreatment with 600 nmol α,β-methylene ATP did not significantly inhibit ($P>0.05$) nociceptive responses evoked by hindpaw injection of either bradykinin (1 nmol) or formalin (0.5%, 0.1 ml) (Figure 5).

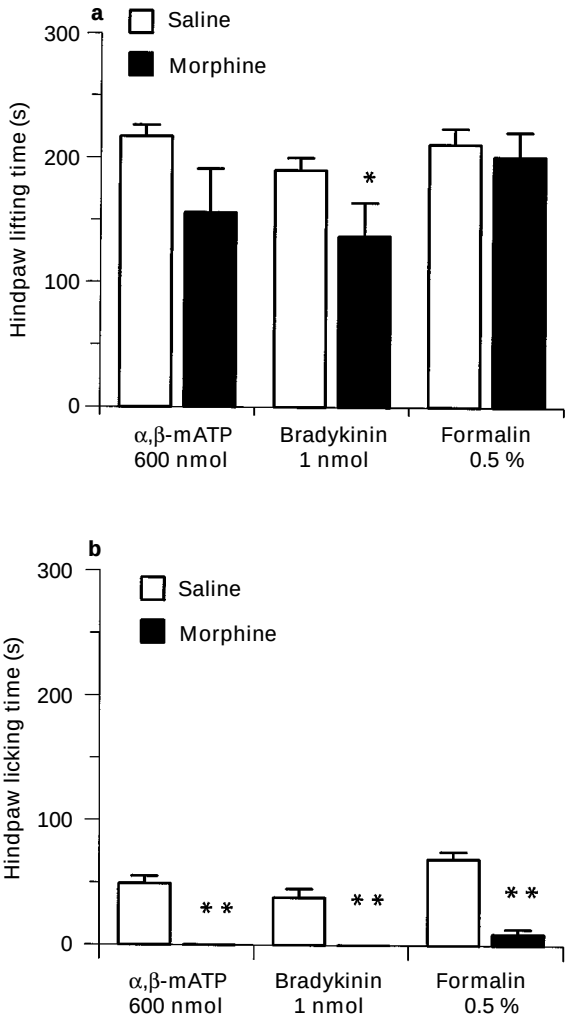

Figure 4 The effect of i.v. morphine pretreatment on hindpaw lifting (a) and hindpaw licking (b) observed during the 5 min period immediately following subplantar administration of α,β-methylene ATP (600 nmol), bradykinin (1 nmol) or formalin (0.5%, 0.1 ml). Rats were pretreated with morphine (3 mg kg^{-1}, i.v.) or an equivalent volume of saline vehicle (Control; 2 ml kg^{-1}, i.v.) 10 min before subplantar algogen administration. Columns indicate mean±s.e.mean ($n=8$ animals in each group). *$P<0.05$ compared with control bradykinin-induced hindpaw lifting, **$P<0.01$ compared with the appropriate control group.

The effect of α,β-methylene ATP after local capsaicin pretreatment

In order to gain an insight into the nature of the sensory neurones stimulated by α,β-methylene ATP, animals were given, under general anaesthesia, a single subplantar injection of either capsaicin (100 μg) or vehicle 3 h before stimulation of nociceptive activity by subplantar injection of 600 nmol α,β-methylene ATP, 1 nmol bradykinin or formalin (0.5%, 0.1 ml). Local capsaicin treatment in this way has been shown in this laboratory to reduce the sensitivity of the treated hindpaw to noxious mechanical (but not thermal) stimuli (data not shown). In all cases nociceptive activity was almost totally inhibited in animals that had received the capsaicin pretreatment when compared with responses observed in vehicle treated animals (Figure 6). In contrast, subplantar injection of formalin (0.5%, 0.1 ml) into the contralateral hindpaw of capsaicin-pretreated animals invariably resulted in an immediate and vigorous hindpaw shaking, biting and licking response (data not shown).

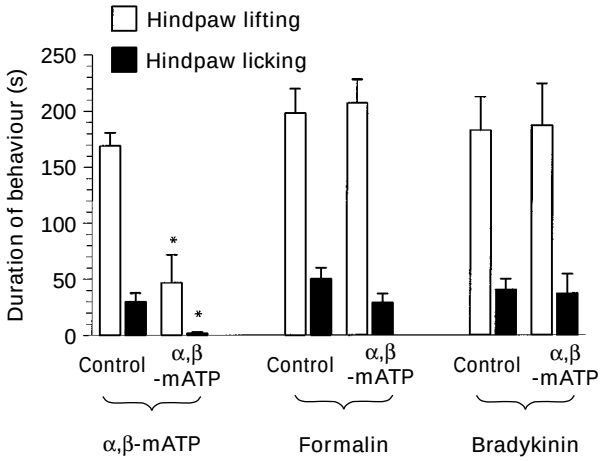

Figure 5 The effect of subplantar α,β-methylene ATP pretreatment on hindpaw lifting and hindpaw licking elicited by subsequent subplantar injection of either α,β-methylene ATP (600 nmol), formalin (0.5%, 0.1 ml) or bradykinin (1 nmol). Animals were pretreated with α,β-methylene ATP (600 nmol) or phosphate-buffered saline vehicle (Control) 30 min before stimulation of nociceptive activity by algogen administration. Results show paw lifting and licking behaviours recorded (in s) during the 5 min period after the second subplantar injection and the columns represent the mean + s.e.mean ($n=5$ animals in each group). $^*P<0.01$ compared with control.

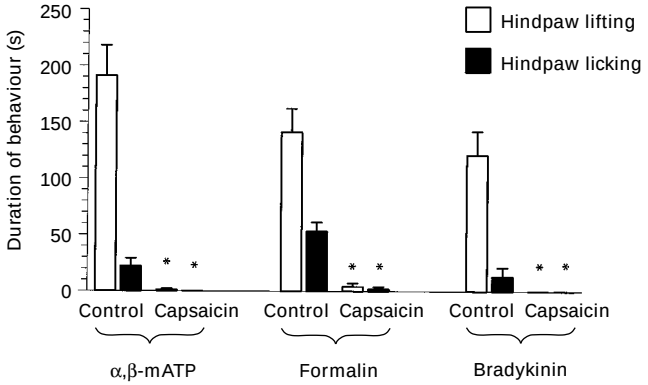

Figure 6 Hindpaw lifting and licking activity evoked by subplantar injection of either α,β-methylene ATP (600 nmol), formalin (0.5%, 0.1 ml) or bradykinin (1 nmol), in rats that had received 3 h pretreatment with either capsaicin (100 μg, subplantar) or vehicle (Control; 0.1 ml, subplantar). Columns represent nociceptive activity observed in the 5 min period immediately following algogen administration and are the mean + s.e.mean ($n=6$ animals in each group). $^*P<0.01$ compared with the appropriate control group.

Discussion

Whilst the role of ATP in nociception has been previously investigated in conscious animals, such studies have focused principally on the effects of currently available, non-specific, P2 receptor antagonists in established pain models. For example, systemic administration of suramin has been shown to display antinociceptive activity in the mouse hot-plate and acetic acid-induced writhing tests (Ho *et al*., 1992), whilst intrathecal administration of either suramin, Evans blue, Trypan blue or Reactive blue 2 reduces nociceptive responses in both the rat tail-flick and formalin tests (Driessen *et al*., 1994). However, interpretation of these results in the context of the importance of ATP in the transmission of nociceptive information is tempered by the non-selectivity (activity at P2X vs P2Y receptors) and the poor specificity (activity at non-P2 and other receptors) of the antagonists employed (see Humphrey *et al*., 1995). In the present study we have used the selective P2X receptor agonist, α,β-methylene ATP (see Humphrey *et al*., 1995), to demonstrate that activation of P2X receptors present on peripheral sensory nerves results in nociception in conscious animals.

Subplantar α,β-methylene ATP injection elicited a transient episode of hindpaw lifting and licking activity comparable to that evoked by subplantar bradykinin administration. Unlike the biphasic profile of nociceptive responses displayed by rats after hindpaw formalin administration, as seen in these experiments and demonstrated previously (e.g. Dubuisson & Dennis, 1977; Abbott *et al*., 1995), α,β-methylene ATP did not induce a late-developing phase of hindpaw lifting and licking. Nonetheless, the acute hindpaw guarding, lifting and licking behaviours observed in animals injected subplantar with α,β-methylene ATP were qualitatively indistinguishable from those observed following subplantar administration of bradykinin or dilute formalin solution, both of which are known to elicit acute, overt nociception when injected subcutaneously in rats and man (Dubuisson & Dennis, 1977; Manning *et al*., 1991; Hong & Abbott, 1994). Taken together, these findings indicate that α,β-methylene ATP-evoked spontaneous hindpaw lifting and licking responses represent a behavioural manifestation of acute nociception in conscious rats. This is consistent with studies in man demonstrating that ATP, applied either topically to a blister base or by intradermal injection, causes the sensation of intense pain (Bleehen & Keele, 1977; Coutts *et al*., 1981), although it was not determined whether ATP acts upon P2X or P2Y receptors to mediate this effect.

The apparent preferential inhibition of hindpaw licking over hindpaw lifting by morphine in the present experiments may indicate a difference in the intensity and/or quality of the nociception represented by the manifestation of each behaviour. *Prima facie*, it may seem logical that hindpaw lifting, being less susceptible to inhibition by morphine, is the behavioural product of a noxious stimulus more intense than that which underlies hindpaw licking (e.g. see discussion in Parsons & Headley, 1989). However, paradoxically, in the late phase of the formalin test in which morphine displays a similar profile of activity to that seen in this study, hindpaw lifting activity has been claimed to represent the manifestation of nociception less intense than that indicated by hindpaw licking (Coderre *et al*., 1993). It would seem important to consider both hindpaw lifting and licking in order to gain a full appreciation of the algogenic effects of substances administered by the subplantar route and the inhibition of such responses with analgesics.

We have provided evidence that subplantar administration of α,β-methylene ATP causes paw lifting and licking behaviours as a consequence of hindpaw sensory nerve stimulation, since pretreatment of ipsilateral hindpaws with the local anaesthetic bupivacaine effectively blocked nociceptive activity. Furthermore, given that capsaicin selectively desensitizes small diameter primary afferent neurones, the abolition of nociceptive responses in capsaicin pretreated animals implies that all sensory information relevant to the manifiestation of these behaviours is conveyed to the CNS by Aδ and C fibres. We presume that α,β-methylene ATP stimulates primary afferent neurones by activation of P2X receptors, since obvious non-specific or indirect mechanisms do not seem to be involved. Thus, equi-osmolar (6 mM) solutions of α,β-methylene ATP and ADP, of approximately equal proton concentration (pH 6.1 and pH 6.2, respectively) elicited markedly differing behavioural responses after subplantar injection, suggesting that nociception is not simply a function of pH or osmotic effects. It also seems unlikely that inflammation triggered by subplantar α,β-methylene ATP administration accounts for the nociceptive behaviour seen in this study. Thus, unlike bradykinin, α,β-methylene ATP did not cause hindpaw swelling at a time point (i.e. 5 min after subplantar injection) when nociceptive responses were most apparent, suggesting that the two phenomena are not causally related. However, a delayed hindpaw swelling was evident 30 min after subplantar administration of 600 nmol α,β-methylene ATP (unpublished

observations), in accordance with the inflammatory effects of this P2X receptor agonist in the mouse (Ziganshina *et al*., 1996).

In view of the fact that rat dorsal root ganglia contain mRNAs encoding most P2X receptor subunits (i.e. all except $P2X_7$) (Collo *et al*., 1996) and that α,β-methylene ATP acts preferentially at P2X receptors (Burnstock & Kennedy, 1985), our data are entirely consistent with the concept that subplantar α,β-methylene ATP stimulates P2X receptors present on the peripheral terminals of small diameter sensory neurones in the rat hindpaw, resulting in the manifestation of behavioural nociception. Non-selective actions at either P1 or P2Y receptors are unlikely to contribute significantly to α,β-methylene ATP-evoked nociception, since subplantar administration of adenosine and ADP, agonists at each of these receptors, did not elicit substantial hindpaw lifting and licking activity. Similarly, subplantar administration of the non-selective P2Y receptor agonist, 2-methylthio ATP (2-meSATP; 600 nmol), did not evoke nociception in conscious rats (unpublished observations). The comparative efficacies of subplantar α,β-methylene ATP, ATP and 2-meSATP at stimulating nociceptive responses may seem surprising given that both ATP and 2-meSATP are more potent than α,β-methylene ATP as agonists at P2X receptors (Valera *et al*., 1994; Chen *et al*., 1995; Khakh *et al*., 1995). However, the rapid hydrolysis of both ATP and 2-meSATP by cell surface ectoATPase enzymes effectively reduces the potency of these agonists on P2X receptors in multicellular preparations, whilst α,β-methylene ATP is resistant to deactivation in this manner (Humphrey *et al*., 1995; Kennedy *et al*., 1996). Therefore it is likely that the limited efficacy of ATP and 2-meSATP in these experiments is a reflection of marked ectoATPase activity present in the subcutaneous tissue of the rat hindpaw.

The identity of the putative P2X receptor(s) stimulated by subplantar α,β-methylene ATP is unknown. However, based on the known pharmacological characteristics of recombinant P2X receptors, it is possible to deduce tentatively that the receptor involved contains $P2X_1$ and/or $P2X_3$ subunits, since these render functional receptors sensitive to α,β-methylene ATP (Valera *et al*., 1994; Chen *et al*., 1995; Lewis *et al*., 1995). Clearly, the involvement of P2X receptors can only be demonstrated conclusively by the inhibition of α,β-methylene ATP-evoked nociception with metabolically stable and selective P2X receptor antagonists. Preliminary attempts to inhibit α,β-methylene ATP-evoked nociception in this manner have so far proved unsuccessful. Thus, systemic pretreatment of rats with the non-selective P2 receptor antagonist suramin (100 μmol kg^{-1}, i.v.) either 20 min or 2 h before subplantar α,β-methylene ATP injection did not inhibit nociceptive activity when compared with vehicle pretreated animals (unpublished observations).

The waning of hindpaw lifting and licking responses elicited by subplantar injection of α,β-methylene ATP may in part be due to clearance of the compound from the site of administration by absorption into local blood vessels. Nonetheless it would appear that desensitization of P2X receptors continually exposed to α,β-methylene ATP could also underlie the gradual fade of nociceptive responses. This view is supported by the observation that a second subplantar injection of α,β-methylene ATP is apparently considerably less noxious when administered after the algogenic effects of the initial α,β-methylene ATP injection have diminished. It is of further interest that α,β-methylene ATP does not cause cross-desensitization, indicated by the observation that subsequent hindpaw injections of either formalin or bradykinin elicit full nociceptive responses. In this regard, it is clear that unlike capsaicin, which desensitizes sensory neurones to all noxious chemical stimuli, α,β-methylene ATP effects a specific desensitization of the P2X receptor channel, leaving sensory conduction intact. These experimental findings also provide evidence that acute pain induced by bradykinin or formalin injection does not involve the local release of ATP to any significant degree.

In conclusion, we have demonstrated that subplantar administration of α,β-methylene ATP (and to a lesser extent ATP) results in behaviours which are partially inhibited by morphine and which are indicative of spontaneous nociception in conscious rats. Since such activity is not evident upon subplantar injection of either ADP or adenosine, we propose that α,β-methylene ATP activates P2X receptors present on small diameter sensory neurones in the rat hindpaw to elicit nociceptive activity. Together, the findings of this study support the recent proposal that ATP released during tissue damage can stimulate peripheral sensory nerve terminals to elicit pain sensation (Burnstock, 1996; Burnstock & Wood, 1996). Spontaneous nociception evoked by subplantar injection of α,β-methylene ATP in the rat could provide a means for examining the activity of putative P2X receptor antagonists *in vivo*. It is anticipated that such an approach will assist the quest for selective and metabolically stable compounds with which to test the hypothesis that ATP has an important pathophysiological role in a variety of painful clinical conditions in man.

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