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Peripheral adenosine 5′-triphosphate enhances nociception in the formalin test via activation of a purinergic p_{2X} receptor

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Abstract

The pronociceptive effects of adenosine 5′-triphosphate (ATP) were examined in the low concentration formalin model (0.5%) by coadministration of ATP, ATP analogs (α,β-methylene-ATP and 2-methylthio-ATP) and antagonists (suramin, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid) with formalin and determining effects on the expression of flinching behaviours. Coadministration of ATP (5–500 nmol) with formalin enhanced phase 2 (12–60 min after injection) but not phase 1 (0–10 min after injection) responses. α,β-methylene-ATP (0.5–50 nmol) but not 2-methylthio-ATP (50–500 nmol) produced a similar enhancement of activity, generating an order of potency of α,β-methylene-ATP, ATP $\gg$ 2-methylthio-ATP. This enhancement was primarily expressed in the latter part of phase 2, 30–60 min after injection. Coadministration of suramin 50–500 nmol, a non-selective P_{2X} and P_{2Y} purinoceptor antagonist and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid 5–500 nmol, a selective P_{2X} purinoceptor antagonist, dose-dependently inhibited the augmentation of the formalin response by ATP 50 nmol, but did not reduce the response to formalin itself. Pretreatment for 30 min with higher doses of suramin inhibited the response to formalin (0.5%, 1.5%) and this appeared to be by a systemically mediated action as it was seen following administration into the contralateral paw. The results of this study provide evidence in support of a P_{2X} purinoceptor mediated augmentation of the pain signal by ATP. The delayed time-course of the effect suggests that it may occur in concert with other mediators that are recruited by the inflammatory process, rather than reflecting a direct depolarization of sensory nerves. Other behavioural paradigms may be required to examine the fast onset, direct effect. Suramin appears to exert both local and systemic effects on the expression of pain behaviours in response to formalin.

Keywords: ATP; α,β-methylene-ATP; Suramin; Nociception; Formalin test

1. Introduction

There is an increasing interest in the role of locally released adenosine 5′-triphosphate (ATP) with a subsequent activation of cell surface P_2 receptors in the regulation of the inflammatory response (Dubyak and El-Moatassim, 1993) and in pain initiation at sensory nerve terminals (Burnstock and Wood, 1996). ATP released under inflammatory conditions could originate from a cytosolic source in a number of cells following lysis or hypoxic-stress, or from more discrete stores in cells such as platelets, mast cells, or sensory or sympathetic nerves. ATP has long been known to stimulate sensory nerve endings, producing an algogenic or pain initiating response (Keele and Armstrong, 1964; Bleehen and Keele, 1977). ATP depolarizes sensory neuron cell bodies by activation of cation channels (Jahr and Jessell, 1983; Krishtal et al., 1988; Bean, 1990); a similar action is presumed to occur at the peripheral sensory nerve terminal accounting for the effect on sensory transmission. Recently, P_{2X} purinoceptor subtypes have been cloned, directly implicated in the fast depolarization of sensory neurons by ATP and proposed to play a selective role in nociceptive activation (Chen et al., 1995; Lewis et al., 1995; Burnstock and Wood, 1996).

While a direct activation of sensory neurons may mediate some aspects of pronociceptive effects of ATP, additional indirect mechanisms via interactions with inflammatory mediators or inflammatory cells may occur (Green et al., 1991; Dubyak and El-Moatassim, 1993). The formalin test is a pain model with two distinct components, an initial phase which reflects a direct sensory nerve activation and a later phase which may reflect an inflammatory component (reviewed in Tjølsen et al., 1992). A variation of this test which uses lower concentrations of formalin

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(0.5–1.0%) has recently been used to evaluate pronociceptive effects of adenosine (Karlsten et al., 1992; Doak and Sawynok, 1995). In the present study, we have determined whether the low concentration formalin model can reveal pronociceptive effects of ATP and whether it can be used to ascertain the role of particular P_2 receptor subtypes in pronociception by evaluating the effect of selective agonists (α,β-methylene-ATP, 2-methylthio-ATP) and antagonists (suramin; pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid or PPADS) on the response to formalin (see Section 4 for a consideration of receptor selectivity). Such a model could be useful for examining potential interactions of ATP with other inflammatory mediators in modulating the sensory afferent pain signal.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats weighing 100–200 g (Charles River, Montreal, Canada) were used in all experiments. Rats were housed in groups of 2–3 at a temperature of 22 ± 1°C on a 12/12 h light/dark cycle, with food and water freely available. Rats which received 0.5% formalin were used twice, with an interval of 5–7 days between trials and the second injection into the contralateral hindpaw. Each experiment was completed and controlled for within a given trial and this accounts for the variability in body weights that occurs between the two trials. Groups of 5–7 rats were used in all experiments except where indicated. All procedures were approved by the University Committee on Laboratory Animals.

2.2. Drug injections and behavioural observations

All drugs were injected s.c. into the dorsal side of the hindpaw in a volume of 50 μl. Drugs were coadministered with the 0.5% formalin by dilution into formalin, or administered as a 30 min pretreatment (in saline) either into the ipsilateral or contralateral hindpaw with appropriate control injections of saline. The contralateral paw was considered to be appropriate for evaluating systemic effects, as factors determining absorption and distribution should be comparable. In some instances, 1.5% formalin was used as indicated.

Rats were acclimatized in the $28 \times 28 \times 28$ cm plexiglass observation chamber for 30 min prior to initiating the experiment. Following the s.c. injection, rats were returned to the observation chamber and the number of flinches (episodes of lifting, shaking, rippling of haunch) determined cumulatively at 2 min intervals for 60 min. Two rats in separate chambers were observed at the same time in alternate 2 min bins. Data reported at 4 min intervals in the time-courses represents results from the 2 min observation interval. Responses observed in phase 1 (0–10 min) and phase 2 (12–60 min) were analyzed separately. Subsequently, when effects were noted to occur consistently at certain times, a separate analysis of phase 2A (16–32 min) and phase 2B (36–60 min) responses was made.

2.3. Data expression and statistics

Data are expressed as cumulative responses during the individual 2 min bins for each rat (time-courses), or phase 1, phase 2A or phase 2B intervals (cumulative incidence of behaviours). It was assumed that values in contiguous bins were similar, but no adjustment to numbers was made. Individual values thus represent approximately half of the behaviourally expressed value. Comparisons were made using analysis of variance followed by the Student Neuman Keuls test.

2.4. Drugs

Formalin (37% formaldehyde) and ATP were obtained from Sigma (St. Louis, MO, USA), while α,β-methylene-ATP, 2-methylthio-ATP, suramin and PPADS were obtained from Research Biochemicals International (Natick, MA, USA).

3. Results

3.1. Effects of coadministration of ATP and related agonists with formalin on flinching behaviours

The s.c. injection of formalin 0.5% produced a modest but significant phase 2 behavioural response when compared to saline, but there was no significant phase 1 response (Fig. 1A inset). Coadministration of ATP 5–500 nmol with formalin 0.5% produced no significant change in the phase 1 response, but a dose-related increase in phase 2 responses (Fig. 1A and 2). A similar increase in phase 2 responses was seen with α,β-methylene-ATP 0.5–50 nmol but not with 2-methylthio-ATP 50–500 nmol (Fig. 1B, C and Fig. 2). When phase 2 responses are scored cumulatively, the order of potency for augmentation of formalin responses is α,β-methylene-ATP, ATP $\gg$ 2-methylthio-ATP (Fig. 2). The injection of ATP 50 and 500 nmol and α,β-methylene-ATP 50 nmol in the absence of formalin did not produce any effects that are different from saline injection ($n = 3$ each, cumulative phase 2 scores < 10). The coadministration of ATP 50 nmol with formalin 1.5% still did not increase phase 1 responses (cumulative flinches 13 ± 6 compared to 8 ± 4 for formalin alone, $n = 7$ per group), while phase 2 responses continue to exhibit an increase in the presence of ATP (cumulative flinches 195 ± 22 compared to 110 ± 13 for formalin alone, $n = 7$ per group, $P < 0.01$).

A close observation of the data indicated that the increase in flinching produced by ATP and α,β-methylene-

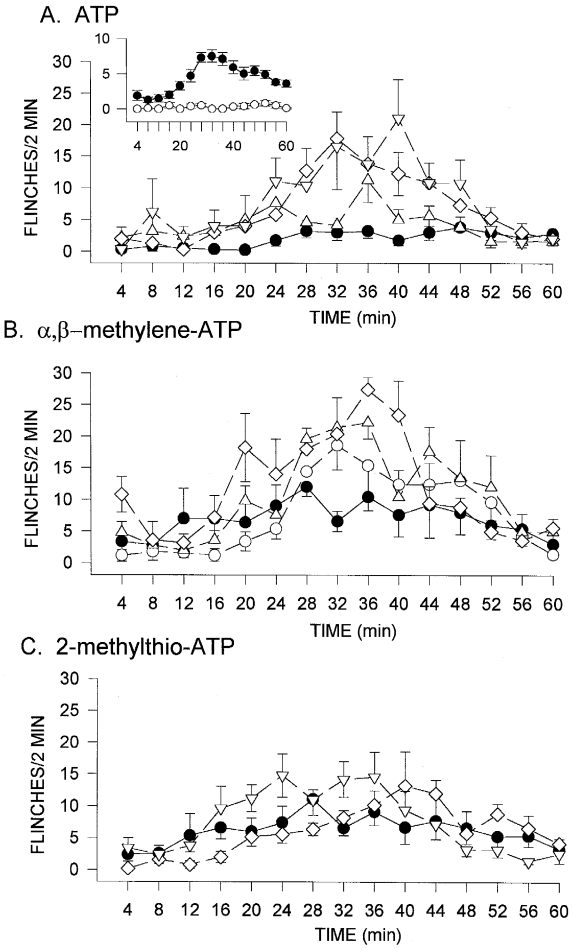

Fig. 1. Time-course of the increase in 0.5% formalin responses by (A) ATP, (B) α,β-methylene-ATP but not (C) 2-methylthio-ATP. Doses in all panels: (○) 0.5 nmol, (△) 5 nmol, (◇) 50 nmol, (▽) 500 nmol (●, formalin 0.5%). Drugs were coadministered with the formalin in a volume of 50 μl. Values in this and all subsequent figures depict mean ± SEM for $n = 6–8$ per group. The inset depicts the pooled formalin 0.5% response for all experiments in this study ($n = 51$) compared to saline (open circle).

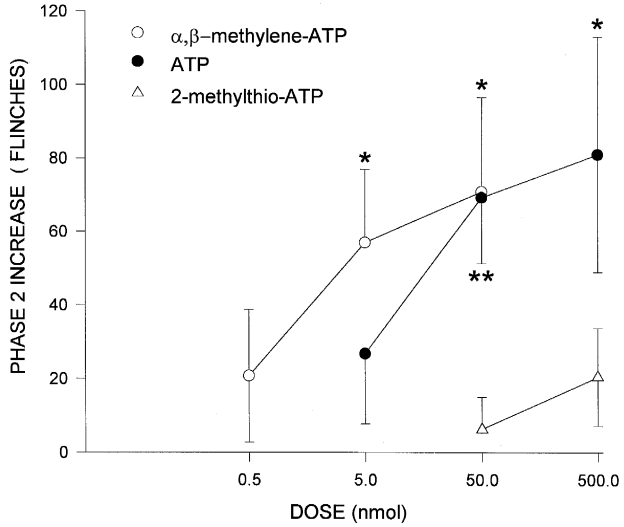

Fig. 2. Dose-dependent increase in the cumulative phase 2 formalin response (16–60 min) following coadministration of ATP and related agonists with 0.5% formalin. Cumulative phase 2 responses for formalin 57 ± 10 ($n = 13$). * $P < 0.05$, ** $P < 0.01$ compared to formalin.

ATP was primarily in the latter part of phase 2 (cf., Fig. 1A and B). In subsequent experiments the early (phase 2A, 16–32 min) and late (phase 2B, 36–60 min) components of this response were evaluated separately and primarily only phase 2B data is presented. No significant changes were observed in any of the other phases.

3.2. Effects of coadministration of ATP antagonists with formalin and formalin / ATP combinations

Coadministration of the non-selective P_{2X} and P_{2Y} purinoceptor antagonist suramin with 0.5% formalin had no significant effect on phase 2 (including phase 2A and phase 2B analyzed separately) flinching behaviours (phase 2: formalin 0.5% 50 ± 8, formalin/suramin 150 nmol

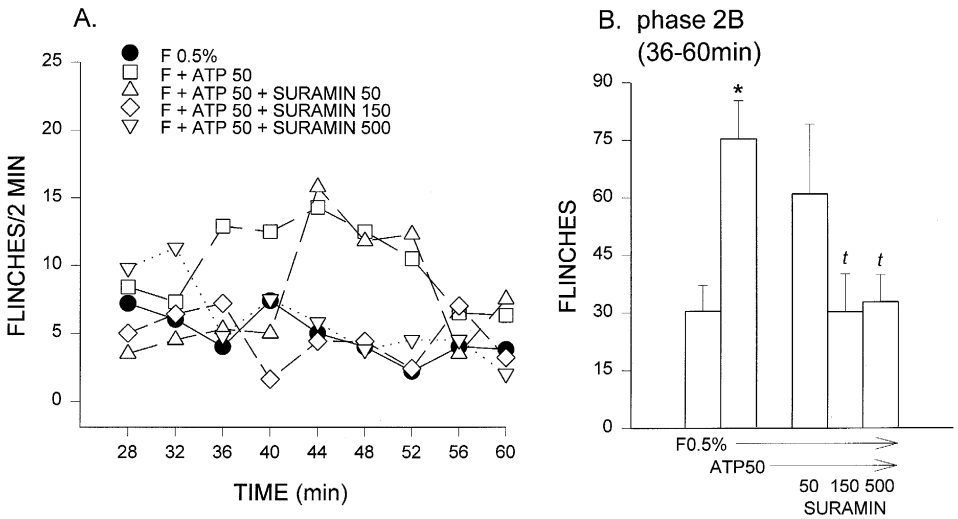

Fig. 3. Dose-related inhibition of phase 2B (36–60 min) responses to formalin (F) 0.5%/ATP 50 nmol by suramin. Values depict means; SEM values omitted in the interest of clarity. Doses are in nmol. * $P < 0.05$ compared to formalin, ^{t} $P < 0.05$ compared to formalin/ATP.

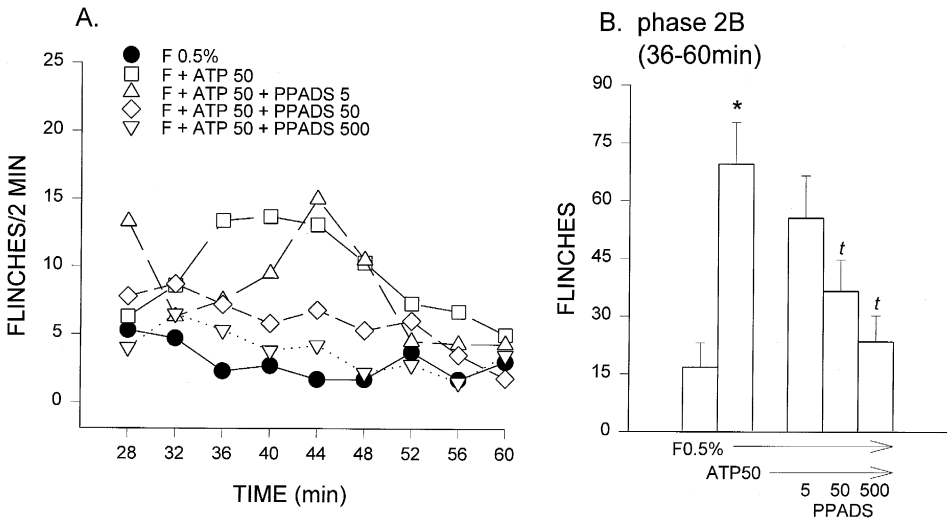

Fig. 4. Inhibition of phase 2B responses to formalin (F) 0.5%/ATP 50 nmol by PPADS. Values depict means; SEM values omitted in the interest of clarity. Doses in nmol. * $P < 0.05$ compared to formalin, † $P < 0.05$ compared to formalin/ATP.

58 ± 15, formalin/suramin 500 nmol 61 ± 4, formalin/suramin 1000 nmol 65 ± 15 flinches, $n = 4–5$). However, coadministration of suramin with formalin 0.5%/ATP 50 nmol produced a dose-dependent reduction in the augmentation of phase 2B responses by ATP (Fig. 3). Coadministration of the selective P_{2X} purinoceptor antagonist PPADS with formalin similarly had no significant effect on the expression of formalin flinching behaviours (50, 500 nmol, $n = 4–5$, data not shown) and inhibited the phase 2B augmentation of the formalin/ATP response (Fig. 4). PPADS was somewhat more active than suramin in inhibiting the ATP response as a lower dose produced a significant reduction in response. In order to verify that the antagonistic effect of PPADS was a locally rather than systemically expressed action, PPADS was administered into the ipsilateral or contralateral hindpaw. Only the ipsilateral administration of PPADS inhibited the formalin/ATP response (Fig. 5).

Both suramin and PPADS (500 nmol each) were also coadministered with 1.5% formalin to determine if the lack of observed intrinsic action against formalin was due to the mildness of the inflammatory stimulus generated by 0.5% formalin. However, neither agent inhibited the expression of phase 2 formalin flinching behaviours at 1.5% formalin (formalin 141 ± 14, formalin/suramin 500 nmol 110 ± 23, formalin/PPADS 500 nmol 118 ± 10, $n = 4–6$). Phase 1 responses also were not altered (data not shown).

3.3. Effect of pretreatment with suramin on the formalin response

In contrast to the lack of effect on formalin responses by the coadministration of suramin, pretreatment with suramin for 30 min produced an intrinsic antinociceptive effect against formalin. Thus, a pretreatment in the ipsilateral hindpaw inhibited formalin-induced behaviours at both

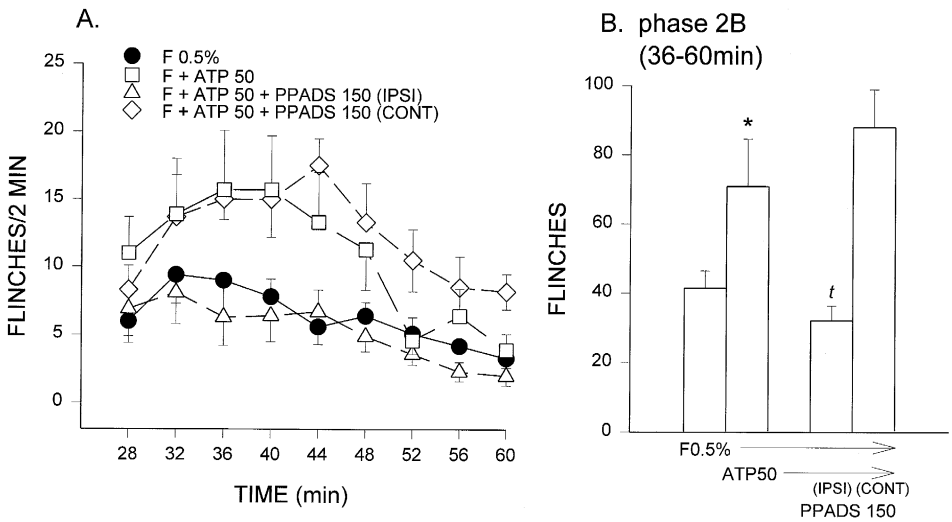

Fig. 5. (A) Time-course of effects of ipsilateral (IPSI) and contralateral (CONT) administration of PPADS 150 nmol on the formalin (F) 0.5%/ATP 50 nmol response, (B) cumulative phase 2B effect. Doses in nmol. ** $P < 0.01$ compared to formalin, † $P < 0.05$ compared to formalin/ATP.

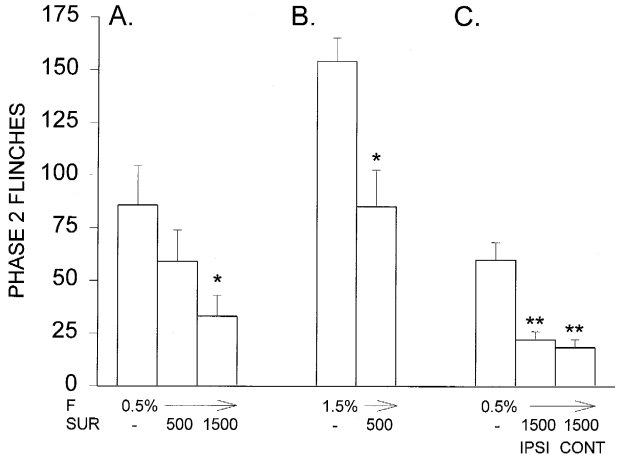

Fig. 6. (A and B) Inhibition of formalin 0.5% and 1.5% responses by a 30 min pretreatment with suramin (SUR) and (C) inhibition of 0.5% formalin response by ipsilateral (IPSI) and contralateral (CONT) pretreatment with suramin. Doses in nmol. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to formalin.

0.5 and 1.5% in the absence of any added ATP (Fig. 6A and B). This effect was due to the systemic absorption of the suramin, as it was also observed following administration of suramin into the contralateral hindpaw (Fig. 6C).

4. Discussion

The present study demonstrates a delayed pain facilitatory (pronociceptive) effect of ATP and α,β-methylene-ATP in a behavioural paradigm in which flinching behaviours are assessed following coadministration with a low concentration of formalin. These agents are inactive in producing this response when administered in the absence of formalin. The enhancement of formalin responses by ATP is blocked both by suramin and PPADS. Both the agonist and antagonist profiles (Fredholm et al., 1994) are consistent with involvement of P_{2X} purinoceptors in the pronociceptive response. It is recognized however, that agonist activities determined here are in an in vivo system where pharmacokinetic issues may confound the observed activity (cf., Kennedy and Leff, 1995) and that both antagonists may have actions in addition to those presumed. Thus, while suramin inhibits both P_{2X} and P_{2Y} mediated responses (e.g., Dunn and Blakely, 1988; Hoyle et al., 1990; Evans et al., 1992), it can also exert other pharmacological actions such as inhibition of ecto-ATPase (Beukers et al., 1995) and of ecto-diadenosine polyphosphate hydrolase (Mateo et al., 1996) and these could contribute to its activity by altering purine availability. PPADS exhibits selectivity for P_{2X} purinoceptors in some preparations (Lambrecht et al., 1992; Ziganshin et al., 1994), but antagonism at P_{2Y} (Boyer et al., 1994) and P_{2U} (Ho et al., 1995) receptors also can occur; inhibition of ecto-ATPase also has been reported (Beukers et al., 1995). An involvement of P_{2Y} and P_{2U} receptors in the responses observed in the present study is unlikely because 2-methylthio-ATP is consistently more potent than α,β-methylene-ATP at those receptors (Fredholm et al., 1994) but is inactive in the present study. It is also unlikely that inhibition of the action of ATP by suramin and PPADS results from inhibition of ATP breakdown to adenosine with a subsequent activation of adenosine A_2 receptors to produce a pronociceptive action as 3,7-dimethyl-1-propargylxanthine, which blocks adenosine A_2 receptor mediated responses (Doak and Sawynok, 1995), does not alter the action of ATP (data not shown).

Pain facilitating effects of ATP have been attributed to activation of ligand gated cation sensitive channels on sensory neurons producing a rapid depolarization (reviewed Burnstock and Wood, 1996). The receptors mediating this action have recently been cloned and characterized as P_{2X2} and P_{2X3} variants (Chen et al., 1995; Lewis et al., 1995). The pronociceptive behavioural effect of ATP observed in this study may not necessarily reflect this direct depolarization, as it exhibits a number of differences compared to the direct depolarization of sensory neurons: (a) Time-course: depolarization of sensory neurons is rapid, being elicited within seconds of application of the ATP (e.g., Bean, 1990), but in the present paradigm, no increase in phase 1 responses (the first 10 min) is observed. Increases in flinching behaviours are seen primarily in phase 2B, at least 30 min following application. (b) Agonist sensitivity: P_{2X} purinoceptors which activate cation channels exhibit sensitivity to 2-methylthio-ATP (Chen et al., 1995; Lewis et al., 1995), yet in our study, no response is seen with 2-methylthio-ATP. This may indicate that in the current in vivo system, 2-methylthio-ATP is subject to degradation by ecto-ATPase, and this limits its activity (Kennedy and Leff, 1995). Alternatively, the profile of the P_{2X} purinoceptors on sensory neurons may be due to it being a heteromer of two P_{2X} purinoceptor variants (Lewis et al., 1995). (c) Requirement for cofactor: ATP and α,β-methylene-ATP are inactive at producing pain behaviours when administered alone and require the co-presence of a low concentration of formalin for flinching behaviours to be expressed. This is in contrast to sensory neuron responses in vitro, where ATP and related agonists produce depolarization when administered as single agents under conditions where indirect actions are less likely (Bean, 1990; Chen et al., 1995; Lewis et al., 1995). The response to ATP may require the presence of a cofactor that is released or synthesized as a result of the inflammatory process and this accounts for its temporal characteristics. The pain signal generated during inflammation is generally regarded to reflect the actions of multiple mediators acting in concert ('inflammatory soup') (Handwerker and Reeh, 1991; Rang et al., 1991) and ATP actions may well represent one example of this. Potential co-agonist candidates include 5-hydroxytryptamine (Bleehen and Keele, 1977), bradykinin (Green et al., 1991) and sub-

stance P (cf., Patra and Westfall, 1996). Activation of ATP receptors on mast cells leads to release of histamine (Dubyak and El-Moatassim, 1993), another potential co-agonist. While ATP receptors involved in this response are P_{2Z} or P_{2Y}/P_{2U} subtypes, the cofactor could be released as a result of the inflammatory process affecting the mast cells rather than the ATP. Both the interactions of ATP with other mediators of inflammation and with inflammatory cells remain to be explored in further detail in the context of explaining the pronociceptive actions of ATP.

In view of the rapid depolarization of sensory neurons repeatedly observed with ATP in electrophysiological studies, the lack of augmentation of phase 1 responses by ATP in this study was surprising. As there was essentially no phase 1 response produced by 0.5% formalin, the possibility that higher concentrations of formalin are required in order to see the effect with ATP was considered, but this did not appear to be the case, as no phase 1 augmentation was seen even with 1.5% formalin. Administration of formalin produces multiple behaviours (Coderre et al., 1993; Abbott et al., 1995), and it may be that nociceptive actions would be observed if other behaviours were assessed. An alternative approach to characterizing this fast component of action in a behavioural paradigm might be to coadminister ATP with selected inflammatory mediators in the absence of formalin and evaluate multiple parameters, as this paradigm reveals fast onset and shorter duration behavioural responses suggestive of pain and hyperalgesia by a number of inflammatory mediators (Hong and Abbott, 1994).

In the present study, the coadministration of suramin and PPADS, in doses that are sufficient to block the effects of exogenously added ATP, exhibits no intrinsic ability to reduce the response to formalin itself. This suggests that ATP is not a major endogenous mediator of the phase 2 inflammatory response following administration of formalin, or that such an involvement is revealed only under certain conditions. It should be noted that the local administration of antagonists to histamine, 5-hydroxytryptamine, prostaglandin E_2, bradykinin and adenosine produces antinociception when coadministered with formalin (reviewed Tjølsen et al., 1992) implicating a local involvement of multiple mediators in the second phase formalin response.

In contrast to the lack of effect with a coadministration protocol, pretreatment with suramin for 30 min produces an intrinsic antinociceptive action. This most likely is a systemically rather than locally mediated effect, because an effect of similar magnitude is observed following the injection of suramin into the contralateral hindpaw. A systemic antinociception has previously been observed in the hot plate and writhing tests; this was blocked by naloxone implicating release of endogenous opioids in this action (Ho et al., 1992). The doses of suramin that are effective in the formalin test (equivalent to 20 mg/kg) are similar to those that are effective in the writhing test (ED_{50} 35 mg/kg) but lower than those that are effective in the hot plate test (ED_{50} 440 mg/kg) (Ho et al., 1992). Antinociception by suramin may result from spinal actions of suramin, as intrathecal administration of suramin produces antinociception in the formalin test, an action most likely due to blockade of spinal pain facilitatory effects of ATP (Driessen et al., 1994). In electrophysiological paradigms using the in situ spinal cord, ATP has been shown to excite both nociceptive and non-nociceptive neurons (reviewed Salter et al., 1993); some of these facilitatory actions may be generated in concert with other agents such as glutamate (Li and Perl, 1995).

The above discussion has focused on pain facilitatory effects of ATP in the periphery and spinal cord mediated by activation of P_2 receptors. It is important to note that ATP also exerts antinociceptive actions due to breakdown to adenosine and subsequent activation of adenosine receptors. Thus, both i.v. ATP (Gomaa, 1987; Kikuta et al., 1990) and intrathecal ATP (Doi et al., 1987) produce antinociception which is blocked by methylxanthines. Electrophysiological studies indicate ATP produces both excitation and depression of wide dynamic range neurons and that the depressant action is blocked by methylxanthines (Salter et al., 1993). This indirect action of ATP contributes to antinociception generated by mechanical vibration (Salter and Henry, 1987) and by transcutaneous electrical nerve stimulation (Marchand et al., 1995). A comprehensive understanding of the multiple ways (peripheral versus spinal and direct versus indirect) in which ATP modulates the transmission of nociceptive information is required in order for a rational development of therapeutic agents which may target this particular receptor system.

Acknowledgements

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