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A novel P_2-purinoceptor expressed by a subpopulation of astrocytes from the dorsal spinal cord of the rat

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1 Astrocytes from the dorsal spinal cord express P_2-purinoceptors which, when stimulated, produce a rise in the intracellular level of free Ca^{2+} ($[Ca^{2+}]_i$). Previously we have found that the P_{2Y} class of receptor is expressed by nearly all astrocytes from the dorsal horn. To determine whether other metabotropic P_2-purinoceptor classes are also present, in this study we investigated the effects of UTP.

2 Application of UTP (1-500 μM, 5-20 s) produced a transient rise in $[Ca^{2+}]_i$ in a subpopulation of astrocytes. The magnitude of the peak increase in $[Ca^{2+}]_i$ was dependent upon UTP concentration and the EC_{50} was found to be 5.2 ± 0.2 μM. Ca^{2+} responses were maximum at 100 μM UTP.

3 The rise in $[Ca^{2+}]_i$ in response to UTP was not affected by removal of extracellular Ca^{2+}. On the other hand, application of the sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin, abolished responses to UTP. These findings indicate that UTP stimulates the release of Ca^{2+} from a thapsigargin-sensitive intracellular pool.

4 The Ca^{2+} response to UTP was unaffected by treatment with pertussis toxin, suggesting that UTP responses may be mediated via a pertussis toxin-insensitive G protein.

5 While all cells tested ($n=52$) responded to the P_{2Y}-purinoceptor agonist, 2-methylthio-ATP, only a subpopulation of astrocytes ($n=67/93$) was responsive to UTP. The presence of UTP-sensitive and UTP-insensitive cells requires the existence of two discrete types of receptor. One receptor, expressed by UTP-insensitive cells, appears to be activated selectively by 2-methylthio-ATP.

6 To investigate whether UTP and 2-methylthio-ATP activate a common type of receptor in UTP-responsive cells, a cross-desensitization strategy was used. Desensitization with prolonged exposure to a high concentration of 2-methylthio-ATP failed to affect responses to UTP and *vice versa*, indicating that receptors activated by UTP are distinct from those activated by 2-methylthio-ATP.

7 The P_2-purinoceptor antagonist, suramin (100 μM), blocked Ca^{2+} responses to UTP and to 2-methylthio-ATP.

8 Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), has been reported to block responses mediated by P_{2X}- and P_{2Y}-purinoceptors in other systems and therefore we investigated its effects on responses to 2-methylthio-ATP and to UTP. PPADS was found to block Ca^{2+} responses to 2-methylthio-ATP in a concentration-dependent manner with an IC_{50} of 0.92 ± 0.1 μM. PPADS also blocked UTP-evoked responses and the IC_{50} was 7.2 ± 1.9 μM. At a concentration of 10 μM, PPADS produced a rightward shift in the dose-response curve for UTP and did not affect the maximum response.

9 Calcium responses evoked by the muscarinic agonist, carbachol, were unaffected either by suramin (100 μM) or by PPADS (50 μM).

10 The present results indicate the presence of a novel class of metabotropic P_{2U}-purinoceptor in dorsal spinal astrocytes. In contrast to P_{2Y}-purinoceptors, the P_{2U}-purinoceptor is expressed only by a subpopulation of astrocytes and its sensitivity to suramin and PPADS distinguish this receptor from P_{2U}-purinoceptors found in other tissues.

Keywords: Fura-2; spinal dorsal horn; P_{2U}-purinoceptors; P_{2Y}-purinoceptors; suramin; PPADS; thapsigargin; UTP; 2-methylthio-ATP; pertussis toxin

Introduction

Adenosine triphosphate (ATP) has been recognized as an intercellular signalling molecule in the peripheral and central nervous systems (Phillis & Wu, 1981; Stone, 1981; Burnstock, 1990; Salter *et al.*, 1993). In the peripheral nervous system, ATP appears to mediate fast excitatory junctional potentials at neuromuscular synapses in the vas deferens (Sneddon *et al.*, 1982) and vascular smooth muscle (Sneddon & Burnstock, 1984; Benham, 1989), and at neurone-neurone synapses in the coeliac ganglion (Evans *et al.*, 1992). In the central nervous system (CNS), neurones in many regions are excited by ATP (Phillis & Wu, 1981; Jahr & Jessell, 1983; Fyffe & Perl, 1984; Salter & Henry, 1985; Illes & Norenberg, 1993) and it has been implicated as a fast excitatory synaptic transmitter in the medial habenula (Edwards *et al.*, 1992). Responses to ATP are not restricted to neurones in the CNS; several other types of cell have been shown to respond to ATP including astrocytes (Neary *et al.*, 1988; Pearce *et al.*, 1989; Bruner & Murphy, 1993a; Salter & Hicks, 1994), microglia (Missiaen *et al.*, 1994; Nörenberg *et al.*, 1994) and oligodendrocytes (Kastritsis & McCarthy, 1993; Salter & Hicks, 1994). The responsiveness of the non-neuronal cells to ATP and its release from synaptic terminals (White, 1985) opens the possibility that it may be a physiological mediator of neurone-to-glia communication (Barres, 1991). ATP and other nucleotides are known to be released with cell damage in the CNS, and these may evoke responses of non-neuronal cells, which are known to be critical in pathogenesis of and recovery from damage (Fedoroff & Vernadakis, 1986). Thus, ATP may have multiple roles in intercellular signalling in the CNS.

ATP has been found to produce increases in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in excitable and in non-excitable cells (El-

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Moatassim *et al.*, 1992). ATP-evoked responses may be mediated by the P_2 type of purinoceptor (O'Connor *et al.*, 1991; El-Moatassim *et al.*, 1992). Five pharmacologically distinct subtypes of P_2-purinoceptor have been proposed (Burnstock, 1990; O'Connor *et al.*, 1991): P_{2X}, P_{2Y}, P_{2Z}, P_{2t} and P_{2U}. The P_{2X}-, P_{2Z}- and P_{2t}-purinoceptors are ligand-gated ion channels which are permeable to Ca^{2+} and other cations (Burnstock, 1990; Bean, 1992). On the other hand, P_{2Y}- and P_{2U}-purinoceptors are metabotropic receptors, and the increase in intracellular Ca^{2+} caused by activating these receptors is produced by releasing Ca^{2+} from intracellular stores (Harden *et al.*, 1990). The division between P_{2Y}- and P_{2U}-purinoceptors has been proposed on the basis of the rank order of potency of ATP and structural analogues, and of UTP (O'Connor *et al.*, 1991). For P_{2Y}-purinoceptors, 2-methylthio-ATP is more potent than ATP, other ATP analogues, or UTP. On the other hand, at P_{2U}-purinoceptors, UTP and ATP are reported to be approximately equipotent and are much more potent than is 2-methylthio-ATP.

In astrocytes, and other non-neuronal CNS cells, a principal mechanism by which signals are transduced and transmitted is by changing $[Ca^{2+}]_i$. Astrocytes from the dorsal spinal cord (Salter & Hicks, 1994) and other CNS regions (Neary *et al.*, 1988; Pearce *et al.*, 1989; Bruner & Murphy, 1993a) have been shown to respond to ATP with a rise in $[Ca^{2+}]_i$. More than 99% of astrocytes respond to ATP and the rank order of potency of adenine nucleotides in producing the increase in $[Ca^{2+}]_i$ in these astrocytes has been found to be 2-methylthio-ATP > ATP $\geqslant$ ADP > AMP = AMP-PCP = 0. The Ca^{2+} response evoked by ATP, ADP or 2-methylthio- ATP has been shown to be due to release of Ca^{2+} from intracellular stores which, together with the rank order of agonist potency, suggest that dorsal spinal astrocytes express P_{2Y}-purinoceptors. The intracellular pool from which Ca^{2+} is released has been characterized as sensitive to thapsigargin and insensitive to caffeine. The intracellular signal transduction pathway leading to release of Ca^{2+} has been shown recently to be the phospholipase $C\beta/IP_3$-pathway with coupling via a G-protein that is insensitive to pertussis toxin (Salter & Hicks, 1995).

We report here that a subpopulation of dorsal spinal astrocytes show Ca^{2+} responses mediated by a subtype of P_2-purinoceptor in addition to and distinct from P_{2Y}-purinoceptors. The additional receptors are activated by UTP and are blocked by the P_2-purinoceptor antagonist suramin, suggesting that they may be P_{2U}-purinoceptors. In contrast to P_{2U}-purinoceptor-mediated responses of other tissues, the Ca^{2+} responses of dorsal spinal astrocytes show a potent and competitive blockade by the recently described P_2-purinoceptor antagonist, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS). Thus, the present findings indicate that there is a novel subtype of P_2-purinoceptor which is expressed by a subpopulation of astrocytes. A preliminary report of part of the results has appeared (Salter *et al.*, 1995).

Methods

Methods for cell culture, for measuring $[Ca^{2+}]_i$ and for drug application have been described in detail previously (Salter & Hicks, 1994).

Cell culture

Primary cultures from the dorsal spinal cord were prepared from foetal Wistar rats (embryonic day 16–18). The foetuses were removed from time-pregnant females (Charles River) that had been anaesthetized with ether and killed by cervical dislocation. Foetuses were transferred to chilled, sterile Hank's buffered salt solution and were then killed by decapitation. The spinal cord was removed and the dorsal half of the cord was dissected by the 'open book' method (Guthrie *et al.*, 1987). The tissue was incubated in trypsin for 25 min at 37°C and triturated to form a cell suspension. Cells were plated on collagen-coated Aclar 33C plastic and grown in minimum essential medium supplemented with 10% foetal bovine serum, 10% heat inactivated horse serum and 1 u ml^{-1} insulin. Aclar 33C plastic transmits infrared, visible and near-visible ultraviolet light making it suitable for use with the ultraviolet-excited fluorescent dye, fura-2. Four days after plating, the culture was treated with 5'-fluoro-2-deoxyuridine (25 $\mu g\ ml^{-1}$) and uridine (50 $\mu g\ ml^{-1}$) for 24 h. On day 4 and 8, two thirds of the culture medium was removed and replaced by fresh media. Cultures were used for recording 6–14 days after plating.

Measurement of intracellular $[Ca^{2+}]$

Intracellular $[Ca^{2+}]$ was measured optically with the Ca^{2+}-sensitive fluorescent dye, fura-2 (Grynkiewicz *et al.*, 1985). To load the cells with fura-2, the culture dish was washed with extracellular recording solution containing (mM): NaCl 140, KCl 5.4, N-2-hydroxyethylpiperazine sulphonic acid (HEPES) 25, glucose 33, $CaCl_2$ 1.3, $MgCl_2$ 1, tetrodotoxin 0.001; pH 7.35, osmolarity 310–320 mosM. The cells were then incubated for 1.5 h at 22°C in extracellular recording solution supplemented with the membrane permeant form of fura-2, fura-2 AM (2–3 μM) and bovine serum albumin (0.5%). Subsequently, the culture dish was washed with extracellular recording solution. With this loading protocol, fura-2 AM was fully de-esterified and more than 95% of the dye was localized in a freely-diffusible cytoplasmic compartment (Salter & Hicks, 1995).

The cultures were placed in a recording chamber on a Nikon Diaphot microscope. Excitation light was produced by a 75 W xenon arc lamp and was passed alternatingly through 340 or 380 bandpass optical filters by means of a mirrored chopper spinning at 60 Hz. The excitation light was transmitted to the culture through a quartz fibre optic cable connected to the epifluorescent adapter of the microscope. Emitted light was passed through a 510-nm barrier filter and light intensity was sampled with a photomultiplier tube detector operating in single-photon counting mode. Adjustable shutters in front of the detector were used to isolate light emitted from a single cell. The fluorescence signal from a cell-free region of the dish was used to determine the background light intensity (Thayer *et al.*, 1988) which was subtracted prior to calculating $[Ca^{2+}]_i$. The ratio of the intensity of light emitted with 340 nm and 380 nm excitations was used to calculate the [free Ca^{2+}] using the formula:

$$[Ca^{2+}] = K^*(R - R_{min})/(R_{max} - R)$$

Where K is the product of the dissociation constant for fura-2 and the ratio of bound to free dye at 380 nm, R_{min} is the minimum ratio corresponding to fully unbound dye and R_{max} is the maximum ratio for the fully bound dye. K, R_{min} and R_{max} were obtained according to the method described by Grynkiewicz *et al.* (1985). Fluorescence signals were acquired, stored and analyzed with hardware and software from Photon Technologies Inc. London, Ontario, Canada. When $[Ca^{2+}]_i$ measurements were not being made, a shutter located in front of the arc lamp was closed to minimize photobleaching of the dye and potential damage of the cells.

Selection of cells

Recordings of $[Ca^{2+}]_i$ were made from individual astrocytes selected by morphological criteria described in Salter & Hicks (1994). As previously shown, such cells express glial fibrillary acidic protein, and have electrophysiological properties characteristic of astrocytes (Salter & Hicks, 1995). To minimize the possibility of Ca^{2+} responses due to signalling between cells, recordings were made from isolated cells in subconfluent regions of the cultures.

Application of drugs

Drugs were applied either via pressure through glass micropipettes with the tip located 10–30 μm from the cell being tested or by exchanging the entire bath volume 4–6 times. Stock solutions of drugs were prepared at 100–10,000× concentration, and were divided into single-use aliquots which were stored in a −70°C freezer. Each aliquot was thawed immediately prior to use and was diluted in the extracellular recording solution.

Experiments with pertussis toxin

In order to investigate the effect of pertussis toxin, cultures were incubated in medium supplemented with 1 μg ml^{-1} pertussis toxin (Sigma) or its vehicle (glycerol 0.5%, Na_2HPO_4 0.5 mM, NaCl 1 mM-final concentration) for 18–20 h at 37°C, just prior to recording. The cultures were subsequently washed with extracellular recording solution, loaded with fura-2 AM, and used as described above. An *in vitro* ADP-ribosylation assay was used to confirm that the treatment with pertussis toxin had caused complete ADP-ribosylation of substrate proteins (Salter & Hicks, 1995).

Data analysis

For each cell in which concentration-response relationships were investigated, the magnitude of peak Ca^{2+} response at each concentration of agonist (A) was calculated as a percentage of the maximum peak response of that cell. The mean of the responses of a number of cells was determined at each concentration and concentration-effect curves were computed by fitting the mean at each agonist concentration with a logistic function: $E = E_{max}/(1 + ([EC_{50}]/[A])^n)$, where E_{max} is the maximum effect. Concentration-inhibition data were collected for PPADS and inhibition curves were constructed by fitting the mean response amplitude at each concentration with the equation $E = E_0/(1 + ([PPADS]/[IC_{50}])^n)$; E_0 is the response amplitude in the absence of PPADS. This equation was sufficient when using 2-methylthio-ATP as an agonist. However, with UTP, the sum of two logistic equations was required to account for the potentiation of responses at low concentrations of PPADS: $E_{max}/(1 + ([PPADS]/a_1)^{n_1}) + (E_0 - E_{max})/(1 + ([PPADS]/a_2)^{n_2})$, where a_1 and a_2 are the concentrations of PPADS at which responses were half maximum and the potentiation was half, respectively. The IC_{50} was determined by solving this equation for [PPADS] when the response amplitude was $E_0/2$. In each case the best fit was determined by the least sum of squares using Sigmaplot software (Jandel Scientific, San Raphael CA, U.S.A.). Values for EC_{50} and IC_{50} from the fitted curves are given ± standard error(s.e.).

Antagonist affinity was estimated by using the general form of the Cheng-Prusoff equation (Leff & Dougall, 1993):

$$K_b = \frac{[IC_{50}]}{(2 + ([A]/[EC_{50}])^n)^{1/n} - 1}$$

where K_b is the dissociation constant. Data for determining values of EC_{50} and n for UTP are reported below; for 2-methylthio-ATP the values used were 0.17 μM and 1.8, respectively (Salter & Hicks, unpublished observations). Values for concentration are reported in μM except where indicated otherwise.

Source of drugs

2-Methylthio-ATP and thapsigargin were obtained from Research Biochemicals Inc., Natick, MA, U.S.A.; fura-2 AM was from Molecular Probes. Suramin was a gift from Dr J Cobby, Bayer Canada, Etobicoke, ON, Canada. PPADS was a gift from Dr G. Lambrecht, Frankfurt, Germany. All other drugs were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results

UTP causes release of intracellular Ca^{2+}

Applications of UTP (1–500 μM, 5–20 s) produced a transient rise in $[Ca^{2+}]_i$ in a subpopulation of dorsal horn astrocytes (Figure 1). The peak level of $[Ca^{2+}]_i$ was reached within 2–5 s and $[Ca^{2+}]_i$ gradually returned to baseline levels over the next 20–40 s. We found that in order to evoke reproducible responses it was necessary to allow an interval of 3–5 min between repeated applications of UTP. With this interval the peak amplitude of the responses declined only 15–20% over up to 4 h of recording.

The magnitude of the Ca^{2+} response to UTP was dependent upon concentration in the range tested, 1–100 μM (Figure 1b, c). The UTP concentration producing 50% of the maximum peak response (EC_{50}) was found to be 5.2 ± 0.2 μM and responses were maximum at 100 μM. The concentration-response relation obtained from our experiments indicate that UTP and ATP have a similar agonist potency in astrocytes (Salter & Hicks, 1994). The sensitivity of these cells to UTP raised the possibility that these cells express P_{2U}-purinoceptors in addition to P_{2Y}-purinoceptors.

To determine whether extracellular Ca^{2+} is required to produce the responses to UTP, we compared responses evoked in extracellular solution containing 1.3 mM Ca^{2+} versus those in solution with no added Ca^{2+} and containing the Ca^{2+} buffer, EGTA (100 μM). As shown in Figure 2a, UTP-evoked Ca^{2+} responses were unaffected by removal of extracellular Ca^{2+}. On average, responses in the Ca^{2+}-free extracellular solution were 95 ± 13% (mean ± s.e.mean) of control values

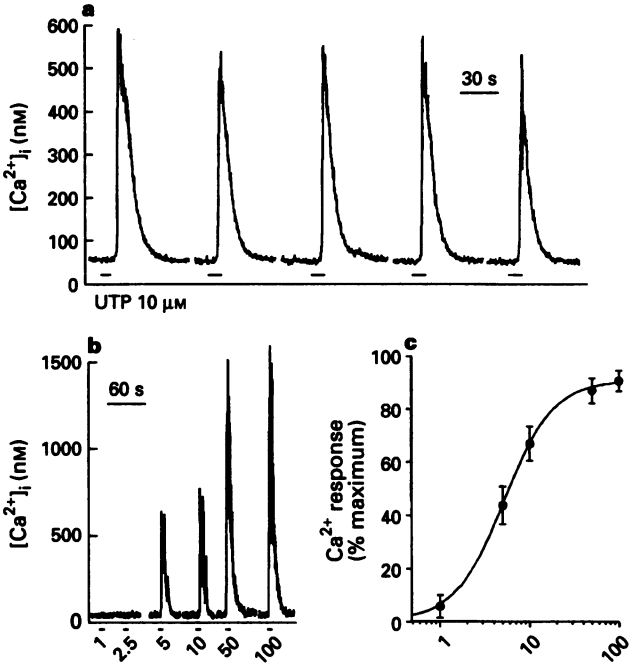

Figure 1 UTP produces a concentration-dependent rise in $[Ca^{2+}]_i$. (a) The traces are records of $[Ca^{2+}]_i$ from an individual astrocyte responding to repeated applications of UTP (10 μM, 10 s). UTP was applied every 5 min to allow for maximum recovery between applications. (b) In a different cell, UTP was applied at concentrations of 1, 2.5, 5, 10, 50 and 100 μM. (c) The graph shows a plot of concentration-response relationship for UTP. Each data point is the mean ± s.e.mean of the peak response to UTP at each concentration for 5–7 astrocytes. The solid curve is the best fit of the means to the logistic equation; the equation of this curve is $95/(1 + (5.2/[UTP])^{1.6})$. In (a) and (b) the periods of UTP application are indicated by the horizontal lines below the $[Ca^{2+}]_i$ recordings. The gaps in the recordings in this figure and all others, indicate periods when fluorescent signals were not sampled as described in the Methods.

($n=12$ cells). These results suggest that extracellular Ca^{2+} is not required but rather that the Ca^{2+} response to UTP is due to release of Ca^{2+} from an intracellular pool.

The intracellular pool from which Ca^{2+} is released was examined by the use of thapsigargin, which is known to inhibit the activity of endoplasmic reticulum Ca^{2+}-ATPases thereby causing depletion of Ca^{2+} (Thastrup, 1990). After application of thapsigargin (1 μM, 2–15 min) the response to UTP was abolished as illustrated in Figure 2b. UTP-evoked responses were similarly affected in all cells tested ($n=7$). Thus, the source of Ca^{2+} released upon stimulation by UTP appears to be a pool in the endoplasmic reticulum that is sensitive to thapsigargin.

Pertussis toxin pretreatment fails to affect UTP-evoked Ca^{2+} responses

For metabotropic receptors that elicit release of Ca^{2+} from thapsigargin-sensitive intracellular stores, the receptors are typically coupled to phospholipase C via heterotrimeric G proteins (Berridge, 1993). In order to determine whether a G protein sensitive to pertussis toxin mediates the response to UTP, we compared responses to a standard application of UTP (10 μM, 10 s) in cells treated with pertussis toxin versus with vehicle alone (Figure 3). Cells treated with pertussis toxin responded to UTP, and the average amplitude of the responses of cells treated with pertussis toxin ($n=12$) was not different from that of cells treated with vehicle ($n=7$). These results suggest that the response to UTP may be mediated by a pertussis toxin-insensitive G protein.

Comparison of astrocytes responding to UTP versus 2-methylthio-ATP

We have reported that dorsal spinal astrocytes express P_{2Y}-purinoceptors on the basis that 2-methylthio-ATP is more potent than is ATP (Salter & Hicks, 1994). The EC_{50} for 2-methylthio-ATP is near 0.2 μM and thus it is also more potent than is UTP. Also, 2-methylthio-ATP causes release of Ca^{2+} from a thapsigargin-sensitive intracellular pool. We therefore considered the possibility that responses to UTP might be mediated via P_{2Y}-purinoceptors. As a first step in investigating the subtype of receptor mediating the response to UTP, we tested cells with 2-methylthio-ATP and with UTP as illustrated in Figure 4. We found that all astrocytes tested responded to 2-methylthio-ATP (0.5–5 μM; $n=52$). On the other hand, only 72% of the astrocytes tested responded to applications of UTP (10–100 μM; 67 of 93 cells). Before being considered non-responsive to UTP (Figure 4b), each cell was tested at a concentration of 100 μM as this was the concentration producing maximum responses in sensitive cells. These data indicate that there are two subpopulations of dorsal horn astrocytes which may be distinguished on the basis of sensitivity to UTP.

Responses to UTP and 2-methylthio-ATP do not cross-desensitize

That there is a subpopulation of cells lacking responses to UTP and yet responsive to 2-methylthio-ATP suggests that the responses are not mediated by a single type of receptor. It is possible that cells in one subpopulation might express receptors that may be activated by either UTP or 2-methylthio-ATP whereas receptors expressed by cells in the other subpopulation might be insensitive to UTP. An alternative possibility is that there might be two types of receptor selectively activated by UTP or by 2-methylthio-ATP, and that cells in the former subpopulation express both types while in the latter subpopulation express only receptors sensitive to 2-methylthio-ATP. In attempting to distinguish between these two possibilities, we challenged UTP-sensitive cells with the one agonist at a sub-maximum concentration and compared the responses before versus after a prolonged application of a high concentration of other agonist (UTP, 100 μM; 2-methylthio-ATP, 5 μM). As illustrated in Figure 5, the Ca^{2+} response evoked by either agonist desensitized gradually during the sustained application. Moreover, we found that responses to test applications of 2-methylthio-ATP persisted after the application of 100 μM UTP (Figure 5a, $n=9$ cells). Conversely, desensitizing with 2-methylthio-ATP failed to affect responses to UTP (Figure 5b, $n=6$ cells). Thus, there was no cross-desensitization, which suggests that two types of receptor are expressed each of which is selectively activated by one or other of the agonists.

Suramin blocks Ca^{2+} responses to UTP and to 2-methylthio-ATP

Suramin, a trypanocidal drug, is known to be a broad spectrum competitive inhibitor of P_2-purinoceptors (Dunn & Blakeley, 1988; Hoiting *et al.*, 1990; Inoue *et al.*, 1991). We found that application of suramin (100 μM) in the bath solution blocked the Ca^{2+} response to UTP as shown in the example in Figure 6a. Washing out the solution containing suramin led to complete recovery of the UTP-evoked increase in $[Ca^{2+}]_i$, indicating the effect of suramin was reversible. Suramin also reversibly blocked the Ca^{2+} response to 2-methylthio-ATP (Figure 6b). Our results indicate the Ca^{2+} response elicited by UTP is mediated by a subtype of P_2-purinoceptor.

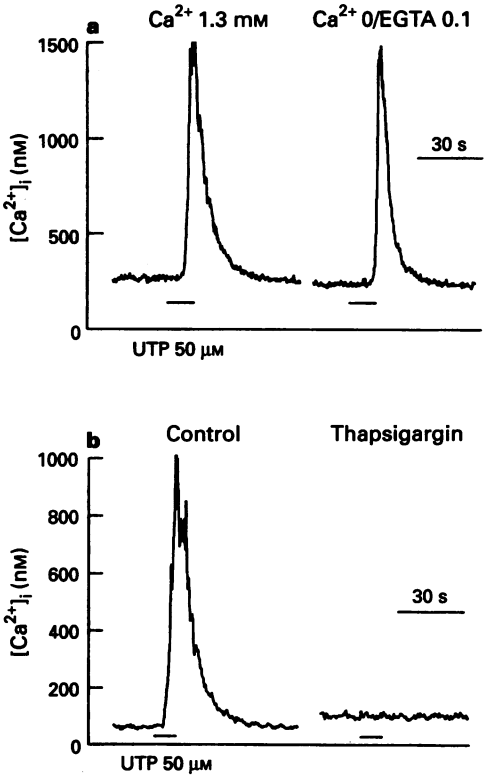

Figure 2 UTP evokes the release of Ca^{2+} from a thapsigargin-sensitive intracellular store. (a) Recordings of $[Ca^{2+}]_i$ from a single astrocyte are shown. On the left, UTP (50 μM, 10 s) was applied as indicated in extracellular recording media containing 1.3 mM Ca^{2+}. In the period between the recordings, the bath solution was replaced with media with no added Ca^{2+} and containing 0.1 mM EGTA. UTP was applied to the same cell after a 10 min interval. (b) The control Ca^{2+} response to the application of UTP (50 μM, 10 s) in another astrocyte is shown on the left. Thapsigargin (1 μM) was applied from a pipette to the cell for a 2 min interval. The record on the right shows the response to the application of UTP 15 min after the application of thapsigargin.

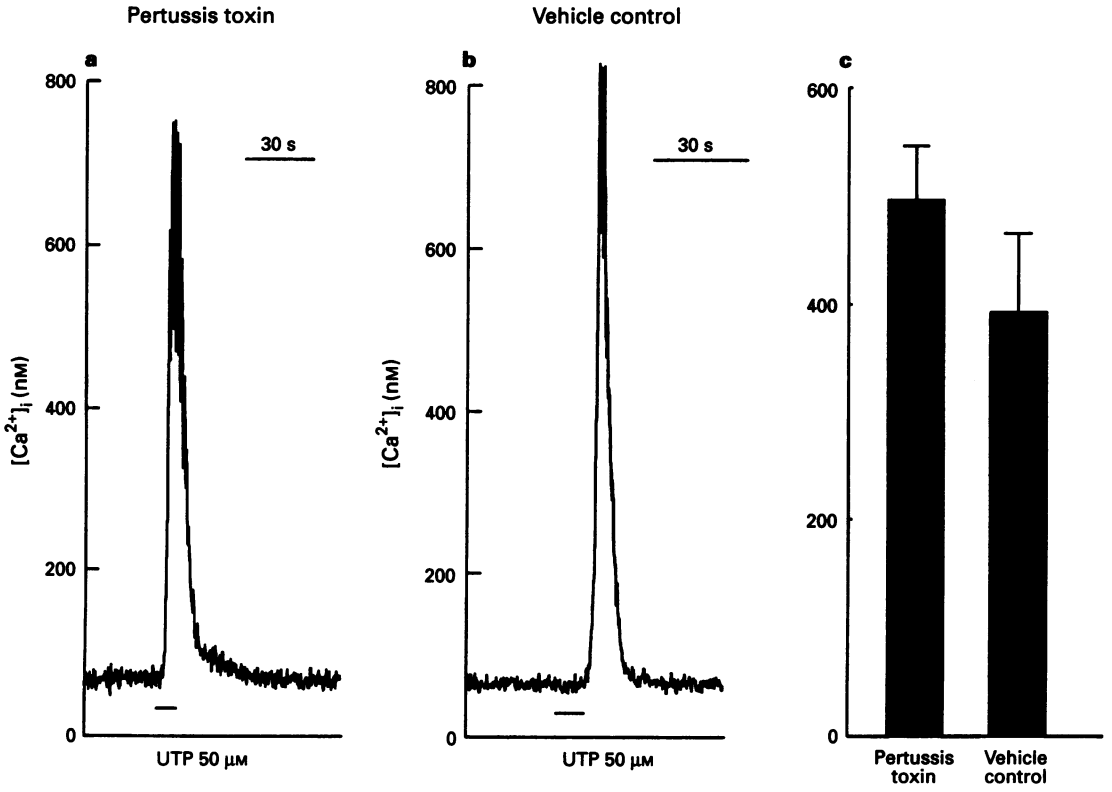

Figure 3 UTP-evoked responses are unaffected by pertussis toxin. Responses to application of UTP (50 μM, 10 s) are shown for a cell treated with pertussis toxin for 20 hours (a) or another cell treated with vehicle alone (b). (c) The peak values of $[Ca^{2+}]_i$ (means ± s.e.mean) are plotted in the histogram. The difference between the means was not statistically significant $P > 0.10$, Student's t test.

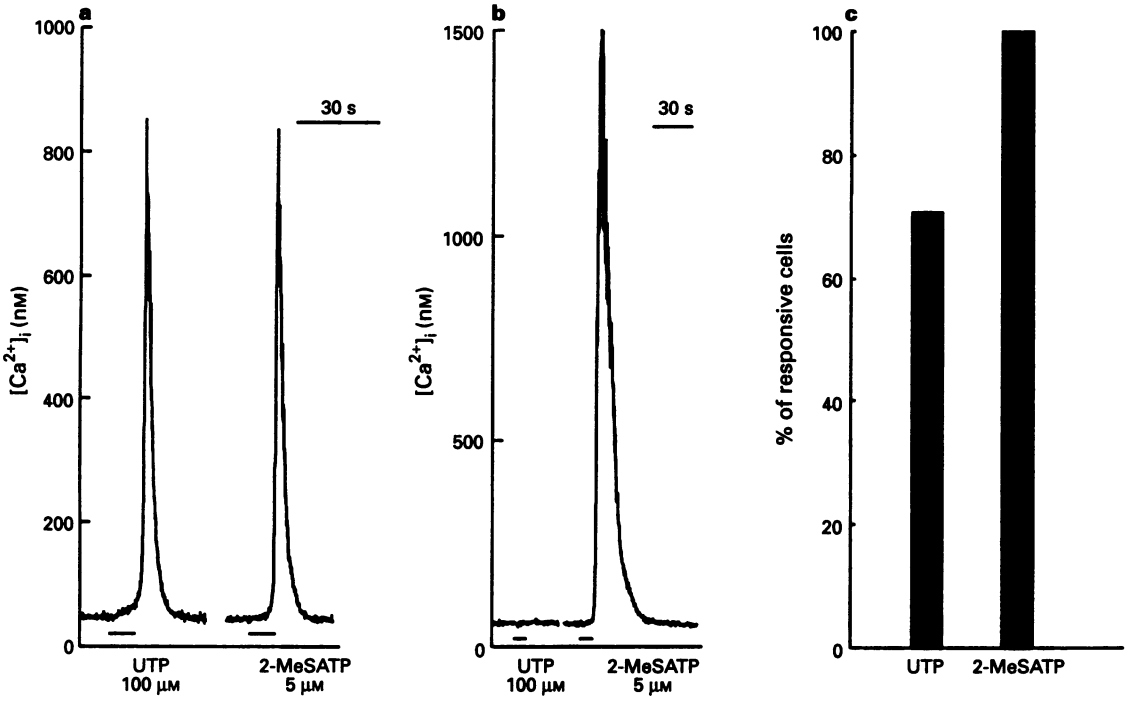

Figure 4 Only a subpopulation of dorsal spinal astrocytes responds to UTP. (a) An example of a cell responding to UTP and to 2-methylthio-ATP. UTP (100 μM, 10 s) and 2-methylthio-ATP (5 μM, 10 s) were applied as indicated. (b) Recordings from a cell that responded to 2-methylthio-ATP but not to UTP. (c) The histogram illustrates the percentage of astrocytes responding to UTP (total, 93 cells) or to 2-methylthio-ATP (total, 52 cells).

Effects of PPADS on Ca^{2+} responses to UTP and to 2-methylthio-ATP

PPADS has been reported to antagonize P_{2X}-purinoceptor-mediated responses in the guinea-pig vas deferens (McLaren *et al.*, 1994), rabbit urinary bladder (Ziganshin *et al.*, 1993) and rabbit isolated blood vessels (Ziganshin *et al.*, 1994), and P_{2Y}-mediated responses in turkey erythrocytes (Boyer *et al.*, 1994). In contrast, it has been found that the response to UTP in the rabbit aorta smooth muscle is unaffected by PPADS (Zigan-

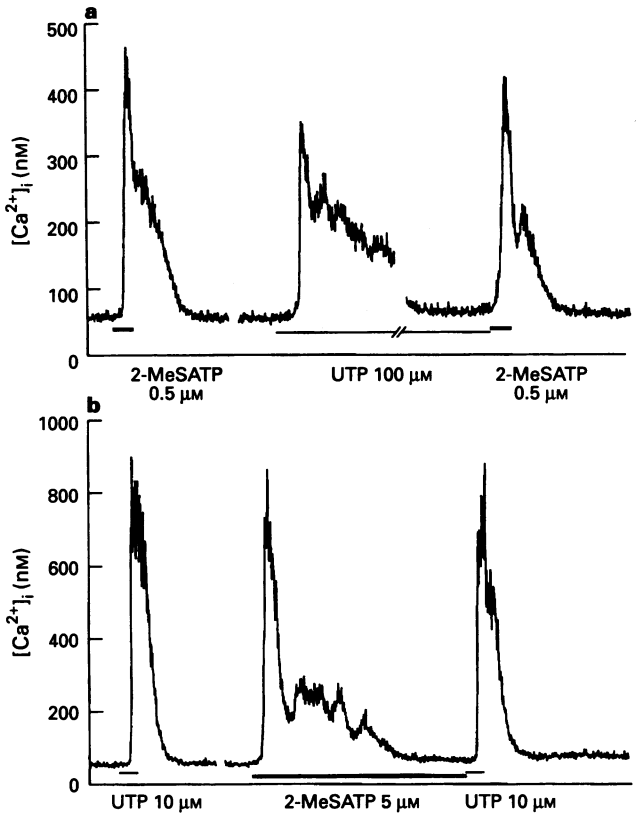

Figure 5 Responses to 2-methylthio-ATP and UTP do not cross-desensitize. (a) 2-Methylthio-ATP (0.5 μM) was applied as indicated by the thick bar horizontal below the trace. After a 5 min recovery period, UTP (100 μM, thin bar) was applied for 7 min. When $[Ca^{2+}]_i$ had returned to the pre-application level, 2-methylthio-ATP was applied again. The double slash indicates a 5 min period during which $[Ca^{2+}]_i$ was not sampled; the UTP application was continuous throughout this period. (b) In another cell, the response to a test application of UTP (10 μM, 10 s) is shown on the left. Five min later, 2-methylthio-ATP (5 μM) was applied continuously until $[Ca^{2+}]_i$ returned to the control level. UTP (10 μM, 10 s) was re-applied as shown on the right.

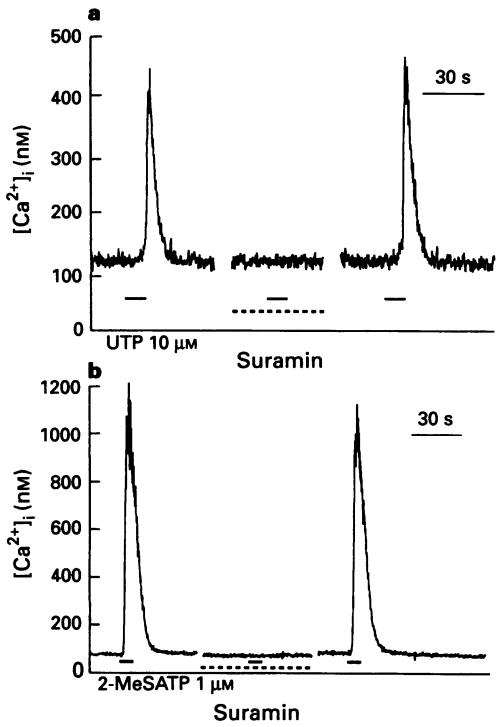

Figure 6 Suramin blocks Ca^{2+} responses to UTP and to 2-methylthio-ATP. (a) UTP was applied before (left), during (middle) or after (right) bath application of suramin (100 μM). In (b) suramin (100 μM) was tested on responses to 2-methylthio-ATP in another cell. The dotted lines below the recordings indicate periods during which suramin was applied.

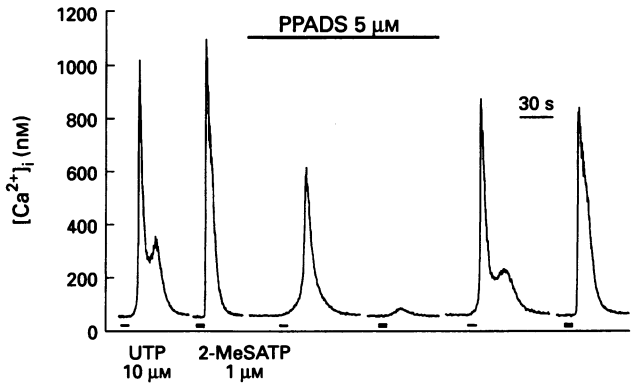

Figure 7 PPADS reversibly depresses Ca^{2+} responses to UTP and 2-methylthio-ATP. Responses of an astrocyte to the application of UTP (10 μM, 10 s) or to 2-methylthio-ATP (1 μM, 10 s) in control extracellular recording media are shown on the left. The bath solution was replaced with extracellular recording media containing 5 μM PPADS, and UTP and 2-methylthio-ATP were re-applied. After washing out PPADS, UTP and 2-methylthio-ATP were applied as shown on the right.

shin *et al.*, 1994) suggesting that it may differentially antagonize P_{2Y}- and P_{2X}-versus P_{2U}-purinoceptor-mediated effects. We therefore investigated the effects of PPADS on responses to 2-methylthio-ATP and UTP. Agonist concentrations of 1 and 10 μM were used, respectively, as these were submaximum and the peak Ca^{2+} responses evoked at these concentrations were approximately equal.

We found that PPADS depressed Ca^{2+} responses to 2-methylthio-ATP and to UTP while having no effect on baseline level of $[Ca^{2+}]_i$ (Figure 7). As illustrated in Figure 8, the decrease of the Ca^{2+} responses was dependent upon the concentration of PPADS and that required to produce 50% inhibition of the response to 2-methylthio-ATP was calculated as 0.92±0.14 μM. The IC_{50} for the blockade of UTP-evoked responses was 7.2±1.9 μM. Thus, the apparent K_b's for PPADS were 0.18 and 4.3 μM for inhibition of responses to 2-methylthio-ATP and UTP, respectively. At concentrations of 10–100 nM PPADS had an additional effect on responses to UTP; these responses were potentiated with the greatest potentiation observed at a PPADS concentration of 10 nM.

As shown in Figure 8d, application of PPADS at a concentration of 10 μM produced a rightward shift in the concentration-response relationship for UTP. The EC_{50} for UTP was increased by PPADS from 5.2±0.5 to 13±2.2 μM whereas the maximum response to UTP was not affected.

Lack of effect of suramin or PPADS on responses to carbachol

In order to examine the selectivity of suramin and PPADS, we tested these compounds on Ca^{2+} responses evoked by the muscarinic agonist, carbachol, which is known to cause release of Ca^{2+} from an IP_3-sensitive intracellular pool (Berridge, 1993). Carbachol (100 μM) elicited reproducible increases in $[Ca^{2+}]_i$ in approximately 30% of dorsal spinal astrocytes. In the presence of suramin (100 μM) responses to carbachol were

not different from the control level (104±4.8%; $n=5$ cells). Similarly, carbachol-evoked responses were not significantly affected (94±6.1% of control; $n=4$) by PPADS (50 μM).

Discussion

In the present study, we demonstrate that astrocytes from the dorsal spinal cord express two discrete P_2-purinoceptors linked to release of stored intracellular Ca^{2+}. The Ca^{2+} responses were evoked by 2-methylthio-ATP or by UTP and we observed that all cells tested were sensitive to 2-methylthio-ATP whereas only about 70% responded to UTP. During prolonged exposure to a high concentration of either ligand, the Ca^{2+} response gradually desensitized but we found no cross-desensitization in the response to the other nucleotide. That Ca^{2+} responses were evoked by the other agonist indicates further that the desensitization could not be attributed to depletion of intracellular Ca^{2+} stores. Responses to 2-methylthio-ATP and to UTP were found to be blocked by suramin. The responses were also blocked by PPADS which was approximately 25 fold more potent in blocking responses to 2-methylthio-ATP than responses to UTP. Neither surmin nor PPADs affected the responses to carbachol. The simplest explanation for these findings is that 2-methylthio-ATP and UTP selectively activate distinct receptors to mediate the Ca^{2+} responses, and furthermore, that the receptors activated by 2-methylthio-ATP are expressed by all of the cells while UTP-sensitive receptors are expressed by only a subpopulation of cells.

We find that the Ca^{2+} responses were evoked by UTP at concentrations similar to those required to produce responses to ATP (Salter & Hicks, 1994), suggesting that UTP and ATP are approximately equipotent. UTP-evoked responses were unaffected by desensitizing concentrations of 2-methylthio-ATP, indicating that 2-methylthio-ATP may be weak or inactive at receptors stimulated by UTP. Thus, the rank order of agonist potency appears to be UTP≈ATP>>2-methylthio-ATP, and the receptors activated by UTP therefore correspond to the established classification for the P_{2U} subtype of receptor (O'Connor *et al.*, 1991). Activation of these receptors in dorsal spinal astrocytes appears to elicit release of Ca^{2+} by generating inositol trisphosphate (IP_3) because UTP-evoked responses were eliminated by thapsigargin which is known to cause depletion of an IP_3-sensitive pool in these cells (Salter & Hicks, 1995). Astrocyte Ca^{2+} responses to UTP were blocked by

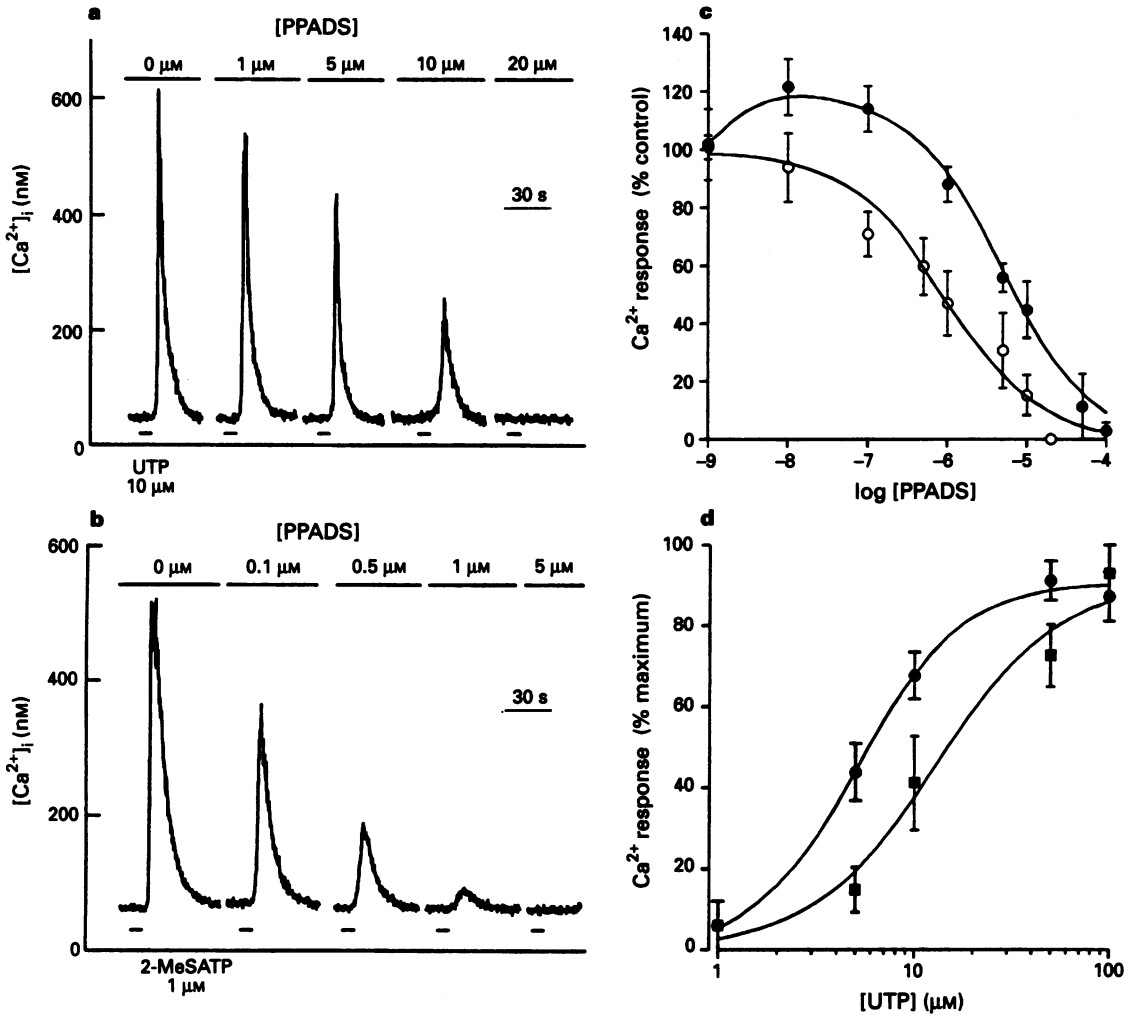

Figure 8 Concentration-dependence of the blockade of responses to UTP and 2-methylthio-ATP by PPADS. (a) UTP-evoked responses are shown for one astrocyte in extracellular recording media containing PPADS in an ascending series of concentrations (0, 1, 5, 10 and 20 μM). (b) For another astrocyte, 2-methylthio-ATP (1 μM, 10 s) was applied with PPADS concentrations of 0, 0.1, 0.5, 1 and 5 μM. (c) Concentration-dependence of effects of PPADS on responses to UTP (●) or 2-methylthio-ATP (○) is shown in the graph. Each data point is the mean±s.e.mean for 5–7 cells. The equations for the smooth curves are $99.5/(1+([PPADS]/0.92)^{0.72})$ for 2-methylthio-ATP and $120/(1+([PPADS]/4.6)^{0.78})-20/(1+([PPADS]/0.002)^{2.5})$ for UTP. (d) The effect of PPADS on the concentration-response relation for UTP is illustrated in the graph. Data are plotted for UTP applied without (●) or with (■) PPADS (10 μM). The equations for the curves are $92/(1+(5.6/[UTP])^{1.6})$ and $91/(1+(13/[UTP])^{1.4})$, respectively. In (c) and (d), the Ca^{2+} responses are expressed as a percentage of the peak response evoked in the absence of PPADS.

suramin which has been reported to inhibit responses mediated by P_{2U}-purinoceptors in DDT1 MF-2 (Hoiting *et al.*, 1990; Sipma *et al.*, 1994) and C2C12 (Henning *et al.*, 1992) cell lines, in rat lactotrophs (Carew *et al.*, 1994) and gondatrophs (Chen *et al.*, 1994). Thus, the blockade by suramin is consistent with P_{2U}-purinoceptors mediating the UTP-evoked response.

Responses to UTP were also blocked in a concentration-dependent and reversible manner by PPADS. The rightward shift in the concentration-response relationship for UTP and the lack of effect on the maximum responses are consistent with the possibility that inhibition by PPADS is competitive. PPADS has been reported to inhibit competitively P_{2X}-purinoceptor-mediated responses in rabbit isolated blood vessels (Ziganshin *et al.*, 1994), rabbit urinary bladder (Ziganshin *et al.*, 1993), rat vagus nerve (Trezise *et al.*, 1994) and guinea-pig vas deferens (McLaren *et al.*, 1994) and P_{2Y}-mediated responses in turkey erythrocytes (Boyer *et al.*, 1994). But the present report is the first evidence that PPADS may competitively inhibit responses mediated by P_{2U}-purinoceptors. The K_b we have found for inhibiting astrocyte Ca^{2+} responses of 4.3 μM is similar to the values of 1–3 μM reported for inhibiting responses mediated by P_{2X}-or P_{2Y}-purinoceptors.

At concentrations 1–2 orders of magnitude lower than those producing inhibition of the Ca^{2+} responses, PPADS caused a small but consistent increase in the UTP-evoked response. At these lower concentrations PPADS had no effect on Ca^{2+} responses evoked by 2-methylthio-ATP indicating that the potentiation was neither attributable to altered intracellular pumping, buffering or sequestration of Ca^{2+} nor to a measurement artefact. Nor could the potentiation be accounted for by a possible partial agonist action of PPADS because at no concentration did it affect the basal level of Ca^{2+}. It remains possible that the potentiation by PPADS might be due to an allosteric interaction with the receptor or to enhanced receptor-effector coupling or to depressing mechanisms, other than those mechanisms mentioned above, that might oppose the rise in Ca^{2+}. The potentiation observed at low concentrations of PPADS may be relevant to calculation of the IC_{50} value. If the potentiation is independent of the inhibition and would add 20% to the peak response at all PPADS concentrations, then the IC_{50} and K_b should be recalculated as 4.6 and 2.7 μM, respectively. PPADS has been found to potentiate P_{2X}-purinoceptor-mediated responses in the guinea-pig vas deferens, indicating that this action is not restricted to P_{2U}-purinoceptors.

The present observations that PPADS competitively inhibits responses mediated by P_{2U}-purinoceptors contrasts with the report from Ziganshin *et al.* (1994) that PPADS has no effect on P_{2U}-purinoceptor-mediated relaxation in the rabbit aorta. PPADS has also been found to depress UTP-evoked increases in cyclic GMP in a neuroblastoma × glioma cell line (Reiser, 1995). These findings suggest that there may be a heterogeneity of P_{2U}-purinoceptors with subtypes distinguishable on the basis of sensitivity to PPADS. That P_{2U}-purinoceptors may be heterogeneous is also suggested by differences in sensitivity to blockade by suramin in various preparations. In addition to our findings that suramin blocks the UTP-evoked Ca^{2+} responses in astrocytes, it has been found to block P_{2U}-purinoceptor-mediated responses in neuronal and muscle derived cell lines (see above). On the other hand, P_{2U}-mediated responses in bovine (Wilkinson *et al.*, 1993; 1994) and rabbit (Chinellato *et al.*, 1994) aorta are reportedly resistant to suramin. P_{2U}-purinoceptors also show differences in G protein coupling. P_{2U}-purinoceptor-mediated responses that are unaffected by pertussis toxin have been reported in pituitary cells (Davidson *et al.*, 1990), as well as in the present study, which indicates coupling via pertussis toxin insensitive G proteins such as G_q/G_{11} (Berridge, 1993). In contrast, pertussis toxin has been found to inhibit, partially or completely, P_{2U}-purinoceptor mediated responses in epithelial cells (Brown *et al.*, 1991), a muscle cell line (Sipma *et al.*, 1994), and with recombinant P_{2U}-purinoceptors from neuroblastoma cells (Erb *et al.*, 1993). This suggests that P_{2U}-purinoceptors may also couple to G_i/G_oG proteins. The differences in sensitivity to antagonists and in signal transduction mechanisms raises the possibility that there are pharmacologically and molecularly distinct subtypes of P_{2U}-purinoceptors.

A cDNA encoding for functional P_{2U}-purinoceptors has been cloned from mouse neuroblastoma cells (Lustig *et al.*, 1993). From the predicted primary amino acid sequence it has been suggested that the cloned P_{2U}-purinoceptor has seven transmembrane spanning domains typical of G-protein coupled receptors. Northern blotting analysis has indicated that the P_{2U}-purinoceptor is widely expressed but the level of expression is much lower in the CNS than in other tissues such as heart, liver or kidney. Given that the majority of dorsal spinal astrocytes respond to UTP and that UTP-evoked responses have also been reported in astrocytes from the cerebral cortex (Bruner & Murphy, 1993b), it is likely that P_{2U}-purinoceptors in the CNS may be abundant. Thus, while it is possible that the molecular identity of the receptor mediating the presently reported Ca^{2+} responses to UTP may be the cloned P_{2U}-purinoceptor, it seems likely that receptor may be a related protein expressed highly in the CNS.

An additional finding in the present study is the blockade of Ca^{2+} responses to 2-methylthio-ATP by PPADS. From the rank order potency found in our prior study (Salter & Hicks, 1994) it appears that responses evoked by 2-methylthio-ATP are mediated by P_{2Y} responses. It has been reported that activation of phospholipase C via P_{2Y}-purinoceptors in turkey erythrocytes is blocked by PPADS (Boyer *et al.*, 1994) at concentrations similar to those used in the present study. But, PPADS does not affect P_{2Y}-purinoceptor-mediated inhibition of adenylate cyclase in C6 glioma cells (Boyer *et al.*, 1994) nor P_{2Y}-mediated relaxation in the rabbit mesenteric artery (Ziganshin *et al.*, 1994). From studies on the potency of ATP analogues it has been recently proposed that there are subtypes of P_{2Y}-purinoceptor (Burnstock *et al.*, 1994). This is supported by comparison of the present results and other recent studies on the sensitivity of P_{2Y}-mediated responses to PPADS.

It is clear that dorsal spinal astrocytes express two pharmacologically distinct metabotropic P_2-purinoceptors, yet both are coupled to release of Ca^{2+} from thapsigargin-sensitive intracellular stores through the activation of a pertussis toxin-insensitive G protein. Co-expression of P_{2Y}- and P_{2U}-purinoceptors has been reported in other tissues including the superior cervical ganglion (Connolly, 1994), bovine aortic endothelial cells (Motte *et al.*, 1993; Wilkinson *et al.*, 1993), PC12 cells (Raha *et al.*, 1993) and aortic smooth muscle (Ziganshin *et al.*, 1994). The functional role for two classes of P_2-purinoceptor on astrocytes remains to be determined. Astrocytes are known to be involved in diverse processes within the CNS and Ca^{2+} signalling in these cells may represent an information processing system which parallels and modulates that in neurones (Cornell-Bell *et al.*, 1990; Dani *et al.*, 1992). Along with a variety of functions, there is well-known diversity of astrocyte phenotype and a number of subclassifications of astrocytes have been proposed (Raff *et al.*, 1983; Miller & Szigeti, 1991; Dave *et al.*, 1991). The differential expression of P_2-purinoceptor subtypes in astrocytes illustrates the heterogeneity in this cell type. It is conceivable that astrocytes in the two subpopulations identified in the present study might have distinct roles in physiological or pathological processes within the CNS.

In conclusion, our results indicate that astrocytes express a subtype of P_{2U}-purinoceptor activated by UTP and blocked by suramin and by PPADS. This receptor is coupled to release of Ca^{2+} from a thapsigargin-sensitive intracellular pool via a pertussis toxin-insensitive G protein. The P_{2U}-purinoceptor expressed by astrocytes is distinct from the P_{2Y}-purinoceptor subtype also expressed by these cells. The presence of two discrete metabotropic P_2-purinoceptors on astrocytes may have significant implications for physiological and pathological processes within the central nervous system. These receptors are potential new targets for the development of novel therapeutic agents for modulating CNS function.

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References

BARRES, B.A. (1991). New roles for glia. *J. Neurosci.*, **11**, 3685–3694.

BEAN, B.P. (1992). Pharmacology and electrophysiology of ATP-activated ion channels. *Trends Pharmacol. Sci.*, **13**, 87–90.

BENHAM, C.D. (1989). ATP-activated channels gate calcium entry in single smooth muscle cells dissociated from rabbit ear artery. *J. Physiol.*, **419**, 689–701.

BERRIDGE, M.J. (1993). Inositol trisphosphate and calcium signalling. *Nature*, **361**, 315–325.

BOYER, J.L., ZOHN, I.E., JACOBSON, K.A. & HARDEN, T.K. (1994). Differential effects of P2-purinoceptor antagonists on phospholipase C- and adenylyl cyclase-coupled P2Y-purinoceptors. *Br. J. Pharmacol.*, **113**, 614–620.

BROWN, H.A., LAZAROWSKI, E.R., BOUCHER, R.C. & HARDEN, T.K. (1991). Evidence that UTP and ATP regulate phospholipase C through a common extracellular 5'-nucleotide receptor in human airway epithelial cells. *Mol. Pharmacol.*, **40**, 648–655.

BRUNER, G. & MURPHY, S. (1993a). Purinergic P2Y receptors on astrocytes are directly coupled to phospholipase A2. *Glia*, **7**, 219–224.

BRUNER, G. & MURPHY, S. (1993b). UTP activates multiple second messenger systems in cultured rat astrocytes. *Neurosci. Lett.*, **162**, 105–108.

BURNSTOCK, G. (1990). Purinergic mechanisms. *Ann. NY Acad. Sci.*, **603**, 1–17.

BURNSTOCK, G., FISCHER, B., HOYLE, C.H.V., MAILLARD, M., ZIGANSHIN, A.U., BRIZZOLARA, A.L., VON ISAKOVICS, A., BOYER, J.L., HARDEN, T.K. & JACOBSON, K.A. (1994). Structure activity relationships for derivatives of adenosine-5'-triphosphate as agonists at P_2 purinoceptors: Heterogeneity within P_{2X} and P_{2Y} subtypes. *Drug Dev. Res.*, **31**, 206–219.

CAREW, M.A., WU, M.L., LAW, G.J., TSENG, Y.Z. & MASON, W.T. (1994). Extracellular ATP activates calcium entry and mobilization via P2U-purinoceptors in rat lactotrophs. *Cell Calcium*, **16**, 227–235.

CHEN, Z.P., LEVY, A., MCARDLE, C.A. & LIGHTMAN, S.L. (1994). Pituitary ATP receptors: characterization and functional localization to gonadotropes. *Endocrinology*, **135**, 1280–1283.

CHINELLATO, A., RAGAZZI, E., PANDOLFO, L., FROLDI, G., CAPARROTTA, L. & FASSINA, G. (1994). Purine- and nucleotide-mediated relaxation of rabbit thoracic aorta: common and different sites of action. *J. Pharm. Pharamcol.*, **46**, 337–341.

CONNOLLY, G.P. (1994). Evidence from desensitization studies for distinct receptors for ATP and UTP on the rat superior cervical ganglion. *Br. J. Pharmacol.*, **112**, 357–359.

CORNELL-BELL, A.H., FINKBEINER, S.M., COOPER, M.S. & SMITH, S.J. (1990). Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science*, **247**, 470–473.

DANI, J.W., CHERNJAVSKY, A. & SMITH, S.J. (1992). Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron.*, **8**, 429–440.

DAVE, V., GORDON, G.W. & MCCARTHY, K.D. (1991). Cerebral type 2 astroglia are heterogeneous with respect to their ability to respond to neuroligands linked to calcium mobilization. *Glia*, **4**, 440–447.

DAVIDSON, J.S., WAKEFIELD, I.K., SOHNIUS, U., VAN DER MERWE, P.A. & MILLAR, R.P. (1990). A novel extracellular nucleotide receptor coupled to phosphoinositidase-C in pituitary cells. *Endocrinology*, **126**, 80–87.

DUNN, P.M. & BLAKELEY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

EDWARDS, F.A., GIBB, A.J. & COLQUHOUN, D. (1992). ATP receptor-mediated synaptic currents in the central nervous system. *Nature*, **359**, 144–147.

EL-MOATASSIM, C., DORNAND, J. & MANI, J.-C. (1992). Extracellular ATP and cell signalling. *Biochim. Biophys. Acta Mol. Cell Res.*, **1134**, 31–45.

ERB, L., LUSTIG, K.D., SULLIVAN, D.M., TURNER, J.T. & WEISMAN, G.A. (1993). Functional expression and photoaffinity labeling of a cloned P2U purinergic receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 10449–10453.

EVANS, R.J., DERKACH, V. & SURPRENANT, A. (1992). ATP mediates fast synaptic transmission in mammalian neurons. *Nature*, **357**, 503–505.

FEDOROFF, S. & VERNADAKIS, A. (1986). *Astrocytes: Cell Biology and Pathology of Astrocytes*. New York: Academic Press.

FYFFE, R.E. & PERL, E.R. (1984). Is ATP a central synaptic mediator for certain primary afferent fibers from mammalian skin? *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 6890–6893.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation Ca2+ indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

GUTHRIE, P.B., BRENNEMAN, D.E. & NEALE, E.A. (1987). Morphological and biochemical differences expressed in separate dissociated cell cultures of dorsal and ventral halves of the mouse spinal cord. *Brain Res.*, **420**, 313–323.

HARDEN, T.K., BOYER, J.L., BROWN, H.A., COOPER, C.L., JEFFS, R.A. & MARTIN, M.W. (1990). Biochemical properties of a P_{2Y}-purinergic receptor. *Ann. N.Y. Acad. Sci.*, **603**, 256–266.

HENNING, R.H., NELEMANS, A., VAN DEN AKKER, J. & DEN HERTOG, A. (1992). The nucleotide receptors on mouse C2C12 myotubes. *Br. J. Pharmacol.*, **106**, 853–858.

HOITING, B., MOLLEMAN, A., NELEMANS, A. & DEN HERTOG, A. (1990). P_2-purinoceptor-activated membrane currents and inositol tetrakisphosphate formation are blocked by suramin. *Eur. J. Pharmacol.*, **181**, 127–131.

ILLES, P. & NORENBERG, W. (1993). Neuronal ATP receptors and their mechanism of action. *Trends Pharmacol. Sci.*, **14**, 50–54.

INOUE, K., NAKAZAWA, K., OHARA-IMAIZUMI, M., OBAMA, T., FUJIMORI, K. & TAKANAKA, A. (1991). Selective and competitive antagonism by suramin of ATP-stimulated catecholamine-secretion from PC12 phaeochromocytoma cells. *Br. J. Pharmacol.*, **102**, 581–584.

JAHR, C.E. & JESSELL, T.M. (1983). ATP excites a subpopulation of rat dorsal horn neurones. *Nature*, **304**, 730–733.

KASTRITSIS, C.H. & MCCARTHY, K.D. (1993). Oligodendroglial lineage cells express neuroligand receptors. *Glia*, **8**, 106–113.

LEFF, P. & DOUGALL, I.G. (1993). Further concerns over Cheng-Prusoff analysis. *Trends Pharmacol. Sci.*, **14**, 110–112.

LUSTIG, K.D., SHIAU, A.K., BRAKE, A.J. & JULIUS, D. (1993). Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 5113–5117.

MCLAREN, G.J., LAMBRECHT, G., MUTSCHLER, E., BÄUMERT, H.G., SNEDDON, P. & KENNEDY, C. (1994). Investigation of the actions of PPADS, a novel P_{2X}-purinoceptor antagonist, in the guinea-pig isolated vas deferens. *Br. J. Pharmacol.*, **111**, 913–917.

MILLER, R.H. & SZIGETI, V. (1991). Clonal analysis of astrocyte diversity in neonatal rat spinal cord cultures. *Development*, **113**, 353–362.

MISSIAEN, L., PARYS, J.B., DE SMEDT, H., HIMPENS, B. & CASTEELS, R. (1994). Inhibition of inositol trisphosphate-induced calcium release by caffeine is prevented by ATP. *Biochem. J.*, **300**, 81–84.

MOTTE, S., PIROTTON, S. & BOEYNAEMS, J.M. (1993). Heterogeneity of ATP receptors in aortic endothelial cells - involvement of P_{2Y} and P_{2U} receptors in inositol phosphate response. *Circ. Res.*, **72**, 504–510.

NEARY, J.T., VAN BREEMEN, C., FORSTER, E., NORENBERG, L.O.B. & NORENBERG, M.D. (1988). ATP stimulates calcium influx in primary astrocyte cultures. *Biochem. Biophys. Res. Commun.*, **157**, 1410–1416.

NÖRENBERG, W., LANGOSCH, J.M., GEBICKE-HAERTER, P.J. & ILLES, P. (1994). Characterization and possible function of adenosine 5'-triphosphate receptors in activated rat microglia. *Br. J. Pharmacol.*, **111**, 942–950.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

PEARCE, B., MURPHY, J., MORROW, C. & DANDONA, P. (1989). ATP-evoked Ca^{2+} mobilisation and prostanoid release from astrocytes: P_2-purinergic receptors linked to phosphoinositide hydrolysis. *J. Neurochem.*, **52**, 971–977.

PHILLIS, J.W. & WU, P.H. (1981). The role of adenosine and its nucleotides in central synaptic transmission. *Prog. Neurobiol.*, **16**, 187–239.

RAFF, M.C., ABNEY, E.R., COHEN, J., LINDSAY, R. & NOBLE, M. (1983). Two types of astrocytes in cultures of developing rat white matter: differences in morphology, surface gangliosides, and growth characteristics. *J. Neurosci.*, **3**, 1289–1300.

RAHA, S., DE SOUZA, L.R. & REED, J.K. (1993). Intracellular signalling by nucleotide receptors in PC12 pheochromocytoma cells. *J. Cell Physiol.*, **154**, 623–630.

REISER, G. (1995). Ca^{2+}- and nitric-oxide dependent stimulation of cyclic GMP synthesis in neuronal cell line induced by P_2-purinergic/pyrimidinergic receptor. *J. Neurochem.*, **64**, 61–68.

SALTER, M.W., DE KONINCK, Y. & HENRY, J.L. (1993). Physiological roles for adenosine and ATP in synaptic transmission in the spinal dorsal horn. *Prog. Neurobiol.*, **41**, 125–156.

SALTER, M.W. & HENRY, J.L. (1985). Effects of adenosine 5'-monophosphate and adenosine 5'-triphosphate on functionally identified units in the cat spinal dorsal horn. Evidence for a differential effect of adenosine 5'-triphosphate on nociceptive vs non-nociceptive units. *Neuroscience*, **15**, 815–825.

SALTER, M.W. & HICKS, J.L. (1994). ATP-evoked increases intracellular calcium in cultured neurones and glia from the dorsal spinal cord. *J. Neurosci.*, **14**, 1563–1575.

SALTER, M.W. & HICKS, J.L. (1995). ATP causes release of intracellular Ca^{2+} via the phospholipase $C\beta/IP_3$ pathway in astrocytes from the dorsal spinal cord. *J. Neurosci.*, **15**, 2961–2971.

SALTER, M.W., HO, C. & HICKS, J.L. (1994). P_{2U} and P_{2Y} purinergic receptors coupled to release of intracellular Ca^{2+} in astrocytes from the dorsal spinal cord. *Drug. Dev. Res.*, **31**, 316.

SIPMA, H., DEN HERTOG, A., & NELEMANS, A. (1994). The phospholipase C activating P2U purinoceptor also inhibits cyclicAMP formation in DDT1 MF-2 smooth muscle cells. *Eur. J. Pharmacol.*, **268**, 431–437.

SNEDDON, P. & BURNSTOCK, G. (1984). ATP as a co-transmitter in rat tail artery. *Eur. J. Pharmacol.*, **106**, 149–152.

SNEDDON, P., WESTFALL, D.P. & FEDAN, J.S. (1982). Cotransmitters in the motor nerves of the guinea pig vas deferens: electrophysiological evidence. *Science*, **218**, 693–695.

STONE, T.W. (1981). Physiological roles for adenosine and adenosine 5'-triphosphate in the nervous system. *Neuroscience*, **6**, 523–555.

THASTRUP, O. (1990). Role of Ca^{2+}-ATPases in regulation of cellular Ca^{2+} signalling, as studied with selective microsomal Ca^{2+}-ATPase inhibitor thapsigargin. *Agents Actions*, **29**, 1–2.

THAYER, S.A., STUREK, M. & MILLER, R.J. (1988). Measurement of neuronal Ca2+ transients using simultaneous microfluorimetry and electrophysiology. *Pflügers Arch.*, **412**, 216–223.

TREZISE, D.J., KENNEDY, I. & HUMPHREY, P.P.A. (1994). The use of antagonists to characterize the receptors mediating depolarization of the rat isolated vagus nerve by α,β-methylene adenosine 5'-triphosphate. *Br. J. Pharmacol.*, **112**, 282–288.

WHITE, T.D. (1985). Release of ATP from central and peripheral nerve terminals. In *Purines Pharmacology, and Physiological Roles*. pp. 95–105. ed. Stone, T.W. London: VCM.

WILKINSON, G.F., MCKECHNIE, K., DAINTY, I.A. & BOARDER, M.R. (1994). P2Y purinoceptor and nucleotide receptor-induced relaxation of precontracted bovine aortic collateral artery rings: differential sensitivity to suramin and indomethacin. *J. Pharmacol. Exp. Ther.*, **268**, 881–887.

WILKINSON, G.F., PURKISS, J.R. & BOARDER, M.R. (1993). The regulation of aortic endothelial cells by purines and pyrimidines involves co-existing P_{2Y}-purinoceptors and nucleotide receptors linked to phospholipase C. *Br. J. Pharmacol.*, **108**, 689–693.

ZIGANSHIN, A.U., HOYLE, C.H.V., BO, X., LAMBRECHT, G., MUTSCHLER, E., BAUMERT, H.G. & BURNSTOCK, G. (1993). PPADS selectively antagonizes P_{2X}-purinoceptor-mediated responses in the rabbit urinary bladder. *Br. J. Pharmacol.*, **110**, 1491–1495.

ZIGANSHIN, A.U., HOYLE, C.H.V., LAMBRECHT, G., MUTSCHLER, E., BÄUMERT, H.G. & BURNSTOCK, G. (1994). Selective antagonism by PPADS at P_{2X}-purinoceptors in rabbit isolated blood vessels. *Br. J. Pharmacol.*, **111**, 923–929.

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