British Journal of Pharmacology (1995) 115, 177–185

A study on P_{2X} purinoceptors mediating the electrophysiological and contractile effects of purine nucleotides in rat vas deferens

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1 We have studied both the electrophysiological and contractile effects of the purine nucleotide, adenosine-5'-triphosphate (ATP), as well as a number of its structural analogues as agonists at P_{2X} purinoceptors in the rat vas deferens *in vitro*.

2 Electrophysiological effects were investigated by a whole cell voltage clamp technique (holding potential −70 mV) with fast flow concentration-clamp applications of agonists in single isolated smooth muscle cells. ATP, 2-methylthio adenosine-5'-triphosphate (2-MeSATP) and α,β methylene adenosine-5'-triphosphate (α,β-meATP) all evoked inward currents over a similar concentration range (0.3–10 μM), being approximately equipotent with similar concentrations for threshold effects (0.3 μM). ADP (10 μM) also evoked a rapid current of similar peak amplitude to that seen with ATP (10 μM).

3 α,β-meATP was the most potent agonist in producing contractions of the rat vas deferens whole tissue preparation, with a threshold concentration equal to that in the electrophysiological studies (0.3 μM). However, ATP and 2-MeSATP were at least ten times less potent in studies measuring contraction than in the electrophysiological studies. Furthermore, their concentration-effect curves were shallow with smaller maximal responses than could be achieved with α,β-meATP. ADP, AMP and adenosine were inactive at concentrations up to 1 mM. The rank order of agonist potencies observed for contraction was α,β-meATP>> ATP = 2-MeSATP.

4 Measurement of inorganic phosphate (iP), as a marker of purine nucleotide metabolism in the vas deferens whole tissue preparation, indicated that ATP and 2-MeSATP were rapidly metabolized, whereas α,β-meATP was stable for up to 2 h. Removal of divalent cations prevented breakdown of ATP and 2-MeSATP, suggesting that metabolism involved a Ca^{2+}/Mg^{2+}-dependent enzyme.

5 It appears that in isolated preparations of rat vas deferens, the low potency of ATP and 2-MeSATP can be explained by rapid agonist breakdown by ectonucleotidases. However, this is not the case in the single cell studies where the use of rapid concentration-clamp applications revealed the true potency of the agonists. Under such conditions the three agonists were all equal in potency indicating that the rank order of agonist potencies of α,β-meATP>> ATP = 2-MeSATP is not in fact characteristic of smooth muscle P_{2X}-purinoceptors as commonly believed.

Keywords: ATP; P_{2X}-purinoceptor; vas deferens; ectonucleotidase

Introduction

Adenosine-5'-triphosphate (ATP) is now widely recognized as a co-transmitter with noradrenaline in the sympathetic nervous system (see Burnstock, 1986; von Kügelgen & Starke, 1991). The most convincing evidence in favour of ATP/noradrenaline co-transmission comes from studies on vas deferens (Sneddon & Burnstock, 1984; Sneddon & Westphall, 1984; Stjarne & Astrand, 1984; see also review by von Kügelgen & Starke, 1991). In rat vas deferens, ATP appears to mediate the rapid twitch component of the contractile response to electrical field stimulation, whereas noradrenaline mediates the slower tonic component (French & Scott, 1983; Amobi & Smith, 1987a,b). Evidence is also emerging that ATP can function as a fast synaptic transmitter at neuro-neuronal synapses in autonomic neurones (Evans *et al.*, 1992) and in the central nervous system (Edwards *et al.*, 1992).

Specific cell surface receptors for ATP, termed P_2-purinoceptors (Burnstock, 1978) have been subdivided into P_{2X} and P_{2Y}-purinoceptor subtypes, largely on the basis of rank orders of agonist potencies (Burnstock & Kennedy, 1985). Since this time several other members of the P_2-purinoceptor family have been proposed (Gordon, 1986; see Abbrachio *et al.*, 1993). The original classification (Burnstock & Kennedy, 1985; Kennedy, 1990) classified the P_{2Y}-purinoceptor as a G-protein coupled receptor which could be identified using the potent and selective agonist 2-MeSATP. The recent cloning of the gene for the P_{2Y}-purinoceptor from chick brain has provided confirmation that the receptor is indeed a member of the G-protein superfamily (Webb *et al.*, 1993; see also Barnard *et al.*, 1994). In contrast the P_{2X}-purinoceptor was classified as a ligand gated cation channel, at which α,β-meATP is a potent and selective agonist (Burnstock & Kennedy, 1985; Kennedy, 1990). Since the original proposal P_{2X} and P_{2Y}-purinoceptors have been characterized in functional studies, largely on the basis of the relative potencies of 2-MeSATP and α,β-meATP (Burnstock, 1990; Kennedy, 1990; Abbrachio *et al.*, 1993; Abbrachio & Burnstock, 1994; Fredholm *et al.*, 1994).

However, we have suggested that the low potency of ATP and its shallow concentration-effect curve as a contractile agonist in rat isolated vas deferens, may be in part due to agonist breakdown or removal (Khakh *et al.*, 1994), thereby underestimating its true potency as an agonist at P_{2X}-purinoceptors. In the present study we have compared the electrophysiological actions of ATP and some of its structural analogues as agonists applied using fast flow concentration-clamp in single smooth muscle cells from rat vas deferens, in an attempt to circumvent the problem of agonist breakdown. The findings were compared with those in whole tissue experiments measuring contraction of rat bisected

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isolated vas deferens. In addition we have obtained a measure of agonist breakdown using inorganic phosphate (iP) production as a marker. The findings are discussed in relation to current criteria for characterization of smooth muscle P_2-purinoceptors, particularly of the P_{2X} type, in isolated tissues.

Some of these results have been presented briefly to the British Pharmacological Society (Khakh *et al.*, 1994).

Methods

Single cell studies

Smooth muscle cells were dissociated by a method based on that already described (Clapp & Gurney, 1991). Male Sprague-Dawley rats (50 g) were killed by carbon dioxide asphyxiation and decapitation. This method of euthanasia is approved by the Office Vetrinaire Cantonal of Geneva where the experiments were conducted. Vasa deferentia were rapidly removed and placed in Ca^{2+}/Mg^{2+} free Hanks buffer (HBSS; Gibco). Adherent connective tissue and mesentery were removed and the tissue chopped into pieces approximately 2 mm square. The pieces were immersed in 2 ml of HBSS with papain 41 iu ml^{-1} (Worthington Biochemicals) and then refrigerated for 18 h at 4°C. This solution was then heated to 37°C in a water incubator and after 10 min the tissue pieces were triturated 10–20 times, in the same solution with fire-polished glass pipettes at times 10, 12, 14, 16 and 18 min until smooth muscle cells could be seen (by inspection of 10 μl aliquots under a light microscope). The solution was then centrifuged at 80 *g* for 10 min, the supernatant discarded and the pellet resuspended in 0.8 ml of extracellular buffer (for composition see below). Aliquots of suspended cells (60 μl) were plated onto 12 mm diameter glass coverslips and left for 20 min at 37°C in a cell incubator. All myocytes were used within 3–3.5 h of plating.

Electrophysiological recordings

Recordings were carried by a whole cell voltage clamp technique using 5 MΩ patch pipettes with seal resistancs of between 20–50 GΩ. Extracellular HEPES buffer had the following composition (mM): NaCl 160, $MgCl_2$ 1.0, $CaCl_2$ 2.0, KCl 2.0, D-glucose 11.0, HEPES 10.0. The buffer was continually superfused at a rate of 1–3 ml min^{-1} through the microscope stage bath (vol 0.5 ml). Intracellular pipette solution contained (mM) K-aspartate 160, NaCl 5.0, HEPES 10.0 and BAPTA 11.0. The pH and osmolarity of internal and external solutions were maintained at 7.4 and 300–312 mosmol ml^{-1}, respectively. Currents were filtered (−3 db) with a 3-pole bessel filter, digitised at 0.5–2 kHz and analysed with a axograph software (Axon instruments).

Agonists were applied using the solenoid operated concentration clamp U-tube method of Fenwick *et al.* (1992). The time course of solution changes was estimated by measuring changes in junction potential as a result of U tube application of distilled water. The time to onset was between 20–300 ms and the rise time (10–90%) was invariably between 10–12 ms.

Each agonist was applied at only one concentration to one cell from any one coverslip. Concentration-effect curves are shown as peak amplitude current responses to a 2 s application of agonist recorded from between 4–12 cells as indicated in the text. Antagonist experiments were performed by exposing cells to 100 μM suramin for 2 min in the superfusion system immediately after attaining whole cell voltage clamp and then applying agonist for 2 s via the U tube in the continued presence of suramin. The fast flow agonist solution also contained 100 μM suramin. Subsequently, cells were superfused with suramin-free medium and then a second application of agonist was made to the same cell in the absence of suramin. All cells were voltage clamped at −70 mV, and all recordings made at room temperature (approx. 22–25°C). The waning of the response in the continued presence of agonist was quantified by fitting to a single exponential and expressed in terms of decay constants using Axograph software (Axon Instruments).

Measurement of contractile activity

Sprague-Dawley rats (200–350 g) were killed by stunning and decapitation. Vasa deferentia were rapidly removed and placed in a Petri dish containing gassed (95% O_2, 5% CO_2, pH 7.4) modified Krebs buffer of the following composition (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $MgSO_4.7H_2O$ 0.6, $K_2H_2PO_4$ 1.5, D-glucose 11.1, $CaCl_2$ 1.3 and indomethacin 6 μM. The tissues were freed of connective tissue and adhering fat, and bisected transversely such that each vasa gave two equal preparations, one prostatic and one epididymal, i.e. four preparations from each animal. The preparations were mounted with cotton thread in 4 ml organ baths under 0.5 g resting tension and immersed in gassed Krebs buffer at 37°C. Tension changes were recorded with a Dynamometer UF1 isometric force transducer and the recordings displayed on a lectromed multitrace 8 channel pen recorder. The preparations were equilibrated under 0.5 g resting tension for a period of 30 min with frequent washes during which time tension was maintained at 0.5 g.

Following equilibration, the preparations were exposed to 100 mM potassium chloride (KCl) three times on a 15 min dose interval. Subsequent contractions (increases in tension) to agonists were expressed as a percentage of the mean contraction to the three doses of potassium chloride. Control first concentration-effect curves were determined for α,β-meATP (10 nM–100 μM) using sequential dosing on a 15 min dose interval to all 4 preparations from each animal. The preparations were washed with Krebs buffer after a maximum response had been attained and then left unchallenged for 1 h. Subsequently in one of the four preparations a second concentration-effect curve was determined for α,β-meATP, and a different agonist was tested in each of the other three preparations. The four preparations obtained from each animal were studied in a Latin square design. All data shown are the mean ± s.e.mean from at least four animals.

Measurement of inorganic phosphate

A colourimetric assay was used to measure the production of iP as a reflection of purine nucleotide metabolism. The method is similar to that already described (Lanzetta *et al.*, 1979) which is based on the method of Hess & Derr (1975). Briefly the method uses a mixture of the dye, malachite green (0.045%), and ammonium molybdate (4.2% in 4 M HCl) that changes colour from yellow to green on complexing with iP. Colour changes were measured as absorbance changes at 660 nm in a spectrophotometer (Perkin Elmer). Absorbance values for the samples were converted to nmol of iP by using a standard curve (which was constructed daily with KH_2PO_4 used as the source of iP). Data are presented as changes in μM concentrations of iP.

Experiments were designed to mimic as far as possible the conditions in the whole tissue contraction studies. The only difference was that a HEPES based buffer (for composition see under single cell studies) was used instead of Krebs buffer to circumvent the problem of background phosphate in the bathing medium. All solutions and drugs used in the measurement of iP were made with ultra pure water (> 18.0 MΩ; Elga water purifier). Bisected vas deferens were placed in 4 ml of HEPES buffer oxygenated continually with 100% O_2 at 37°C, pH 7.35–7.4, and left to equilibrate for 30 min before proceeding further. The four preparations from each animal were arranged in a Latin square design such that the three preparations were incubated with either 100 μM ATP, 2-MeSATP or α,β-meATP and the fourth

preparation always served as a time-dependent control with no drug. Samples of buffer (50 μl) were taken 1 min before adding the drug, immediately after adding the drug (time 0), and then every 10 min for up to 2 h.

Drugs

α,β-Methylene adenosine-5'-triphosphate lithium salt (α,β-meATP), ATP, ADP, AMP, adenosine, HEPES and BAPTA were all purchased from Sigma. 2-Methylthio adenosine-5'-triphosphate tetra sodium salt (2-MeSATP) was obtained from Research Biochemicals Inc. All drugs were made freshly and diluted to the relevant concentration in Krebs solution for contraction experiments and in HEPES buffer for voltage clamp studies and iP measurements.

Results

Single cell studies

Initial observations Preliminary studies showed that a 2 s application of ATP (0.3–10 μM) via the U tube fast perfusion system evoked inward currents in all cells tested in a concentration-dependent manner. The ATP-evoked currents waned in amplitude during the application of agonist. A second application of ATP up to 6 min later showed marked diminution in peak current amplitude response compared to the first application, a property here referred to as tachyphylaxis (Figure 1). The waning of the response during the agonist application and the tachyphylaxis following first application was a property of all concentrations of ATP. Consequently only one concentration of agonist was added to a cell from any one coverslip, in an attempt to circumvent these effects.

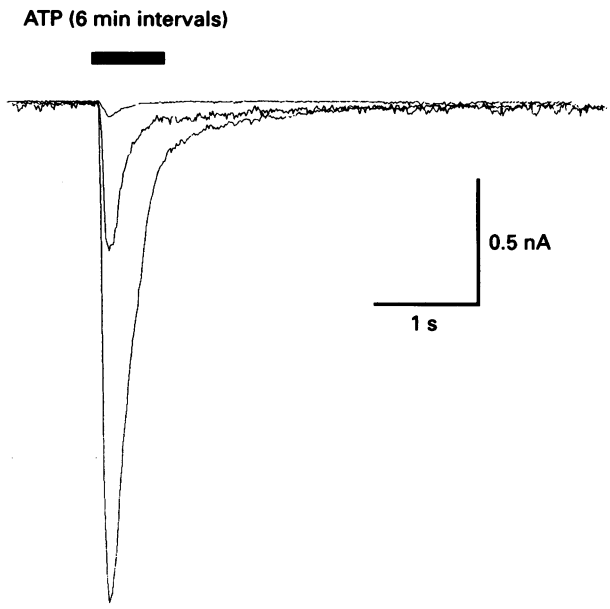

Figure 1 Three superimposed current recordings from a single smooth muscle cell of rat vas deferens in response to U tube concentration clamp application of 3 μM ATP for 0.5 s at 6 min intervals. The response to the second application of 3 μM ATP was markedly smaller, about 30% of initial, and a third application 6 min later produced a response which was only about 10% of the original for this cell. This phenomenon of tachyphylaxis was observed in all cells tested but was not quantified. In this and all further figures the agonist was applied for the time periods as indicated by the solid bar above current recordings.

Concentration-dependence and kinetics of agonist-evoked currents

ATP evoked inward currents over the concentration range 0.1–10 μM. The threshold concentration for responses evoked by ATP was 0.1 μM with a mean peak amplitude response of 65 ± 10 pA ($n = 5$) (see Figure 2). Increasing concentrations of ATP produced larger inward currents (Figures 2 and 3), with progressively shorter rise times, e.g. rise time (10–90% peak) at 0.1 μM was 164.8 ± 27.3 ms ($n = 5$) and at 10 μM 43.5 ± 4.2 ms ($n = 8$; see Figure 2). The decay time of the ATP evoked current could be fitted by a single exponential with time constant values (τ) that were unrelated to agonist concentration (174.3 ± 47 $n = 4$; 221.3 ± 35.6 $n = 11$; and 161.7 ± 14.7 ms $n = 8$ for 1, 3 and 10 μM ATP, respectively). Concentrations of ATP higher than 30 μM were not employed since the rise time at 30 μM ATP was approaching the rise time for the U tube application system. Thus higher concentrations of ATP would have exceeded the limits of adequate solution exchange and underestimated the peak current responses (data not shown). 2-MeSATP was tested over a similar concentration-range to ATP. The threshold for producing a response was between 0.1–1 μM, further the mean peak amplitude current responses were not significantly different from those to ATP at the same concentrations (Figure 3). The response to 2-MeSATP was also rapid (rise time 20.3 ± 3.6 ms at 10 μM; $n = 6$) and the current evoked waned during agonist application (τ = 150 ± 24.2 ms at 10 μM; $n = 6$).

α,β-meATP evoked inward currents with a threshold of between 0.1–0.3 μM. Mean peak amplitude current responses were not significantly different from responses to ATP or 2-MeSATP (Figure 3). However rise times for the α,β-meATP evoked currents were slower than for ATP or 2-MeSATP e.g. 58 ± 9.9 ms at 10 μM compared to 43.5 ± 4.6 and 20.3 ± 3.6 ms for ATP and 2-MeSATP, respectively. Thus it was possible to investigate the actions of α,β-meATP up to 30 μM before the limits of the U tube perfusion system were exceeded; rise time at 30 μM was 34.3 ± 15.8 ms ($n = 3$). The decay constant was slower than for ATP or 2-MeSATP, such that for α,β-meATP (10 μM) τ was 775.8 ± 294.2 ($n = 7$) compared to 161.7 ± 14.7 ($n = 8$) and 150 ± 24.2 ms ($n = 6$) for ATP and 2-MeSATP, respectively (see Figure 2).

ADP (10 μM) evoked an inward current with a similar rise time (58.5 ± 6.2 ms; $n = 4$) and a similar mean peak amplitude (1738 ± 728 pA) current response as that to ATP (1242 ± 364 pA) at the same concentration. However its decay constant of 642.7 ± 77.6 ms ($n = 4$) was significantly longer ($P < 0.05$; Student's t test). AMP and adenosine (both 30 μM) evoked little or no inward current, mean peak amplitudes were 20.3 ± 13.6 and 4.4 ± 4.4 pA, respectively (both $n = 4$); in the same cells ATP was applied to establish cell viability and evoked large inward currents.

Antagonism by suramin

Following a 2 min exposure of cells to 100 μM suramin ATP, 2-MeSATP and α,β-meATP (all at 3 μM) evoked small inward currents of mean peak current amplitude 22.8 ± 13.6, 14.0 ± 10.5 and 18.8 ± 18.8 pA, respectively (all $n = 4$). Replacement of bathing buffer with suramin-free buffer and re-applying agonists 2 min later evoked large inward currents in all cells, with mean peak amplitudes of 1477 ± 519, 1895 ± 661 and 1938 ± 889 pA ($n = 4$ for each), respectively (Figure 4). Likewise the effect of ADP (10 μM) was also abolished by 100 μM suramin ($n = 2$ see Figure 5).

Whole tissue studies

Concentration-dependence of agonists as contractile agents In preliminary studies the actions of ATP and α,β-meATP were studied in epididymal and prostatic preparations to investigate the possibility that differences in agonist potency may exist between the two halves (Sneddon & Machaly, 1992). In

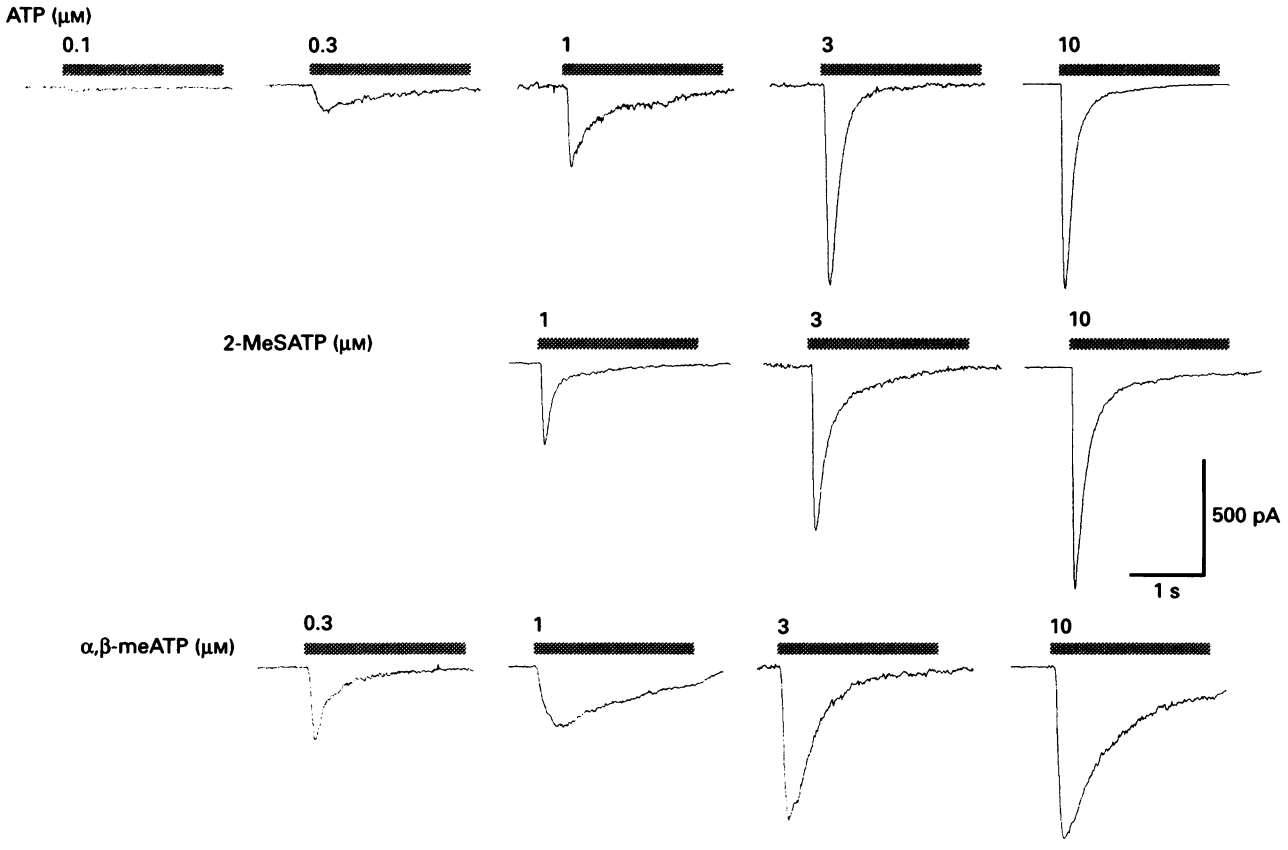

Figure 2 Recordings from single smooth muscle cells of rat vas deferens in response to application of agonists. Upper panels show application of ATP in increasing concentrations as indicated in Figure 1, note threshold is about 0.1 μM. The middle panels show 2-MeSATP evoked inward currents demonstrating concentration-dependency. The lower panels show that α,β-meATP also evoked inward currents in a concentration-dependent manner. Each current recording shown is from a different cell, but is representative of between 4–11 such recordings for each agonist concentration (see Figure 3 and text).

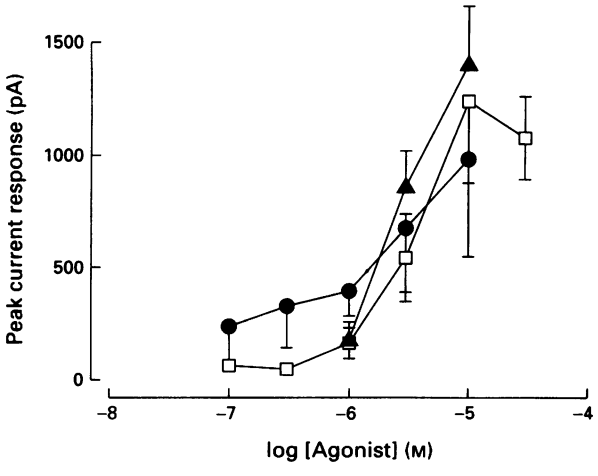

Figure 3 Concentration-effect curves to ATP (□), 2-MeSATP (▲) and α,β-meATP (●) from single smooth muscle cells. Each point on the concentration-effect curves is the mean ± s.e.mean peak inward current for each agonist concentration ($n = 4–11$). All three agonists are approximately equal in potency, ATP had an apparent EC_{50} of about 3 μM. In some cases undirectional error bars are shown for clarity.

such experiments epididymal and prostatic preparations were mounted for isometric tension recording as described in the Methods. Concentration-effect curves (10 nM–100 μM) were determined to α,β-meATP and ATP in both halves. In the case of α,β-meATP concentration-effect curves showed the same threshold concentration for responses (0.3 μM) and similar response amplitudes for both epididymal and prostatic preparations, such that the pEC_{50} for α,β-meATP was 5.34 ± 0.13 and 5.79 ± 0.34 (both $n = 4$; no significant difference at $P = 0.05$) for epididymal and prostatic preparations, respectively. In the case of ATP no EC_{50} value could be calculated since concentration-effect curves were shallow and flat, however mean response amplitudes were not markedly different at all concentrations of ATP examined, such that responses to 1, 10 and 100 μM ATP were 0.6 ± 0.6, 2.4 ± 0.9 and 5.6 ± 1.5 and 1.1 ± 0.9, 2.7 ± 1.5 and 6.0 ± 1.7% of the response to 100 mM KCl for epididymal and prostatic preparations, respectively. Consequently no account was taken of anatomical location other than to pool data from systematically randomized preparations from each animal (see Methods).

ATP induced contractions of rat vas deferens over the concentration range 10 μM–1 mM; threshold concentrations for contraction were about 10 μM. The concentration-effect curve to ATP was shallow (Figure 6) so that at the highest concentration of ATP tested (up to 1 mM) responses were only 17.3 ± 4.4% of the contraction to 100 mM KCl ($n = 4$). Similarly, 2-MeSATP (3–100 μM) displayed a shallow concentration-effect curve, with threshold concentrations for contraction being about 3 μM; the response to 100 μM 2-MeSATP was 17.7 ± 2.3% of the response of 100 mM KCl ($n = 4$). In contrast, α,β-meATP induced contractions of rat vas deferens over the range 0.3–100 μM, with threshold concentrations at 0.3 μM. The concentration-effect curve to α,β-meATP was steep and response amplitude was related to the concentration of α,β-meATP. At the highest concentration of α,β-meATP tested (100 μM) the contractile response was 79.1 ± 11.4% of the response to 100 mM KCl ($n = 4$). However, the concentration-effect curve to α,β-meATP did not appear to reach a well-defined maximal response. ADP,

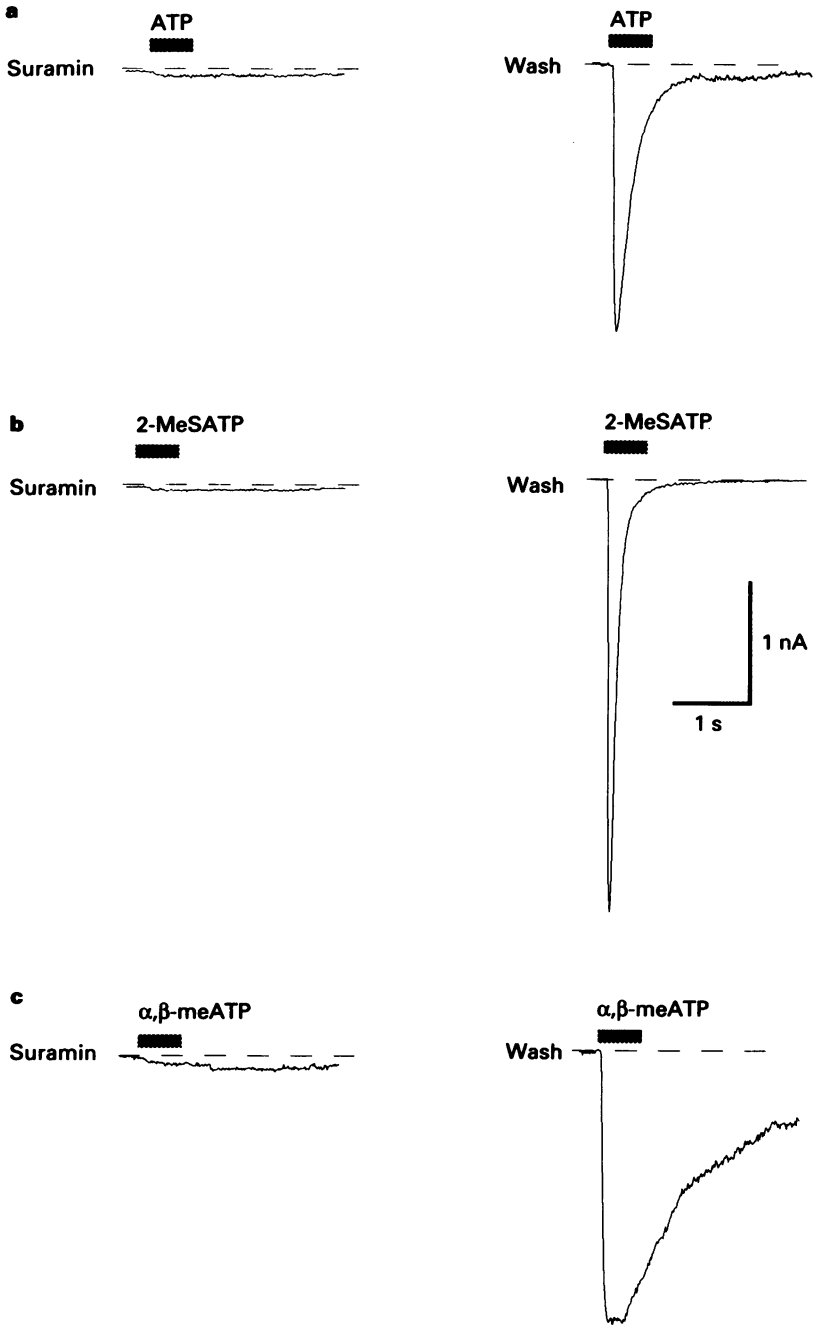

Figure 4 Suramin antagonism of agonist-induced currents in single vas deferens smooth muscle cells. The left hand recordings are in the presence of 100 μM suramin and the right hand traces (indicated as wash) are from the same cells after suramin washout. Large inward currents were recorded in response to application of (a) ATP, (b) 2-MeSATP and (c) α,β-meATP when suramin was absent. All agonist concentrations were 3 μM. Current recordings shown are representative of four such experiments.

AMP and adenosine produced little or no agonist effects at concentrations up to 100 μM.

Measurement of inorganic phosphate

When either ATP or 2-MeSATP (both at 100 μM) were incubated with bisected vasa deferentia and 50 μl samples taken every 10 min and analysed for iP, both compounds led to an increase in iP concentration in a time-dependent manner. After 120 min ATP and 2-MeSATP provided 205.7 ± 24.0 and 216.2 ± 26.9 μM of iP from 100 μM agonist (all $n = 8$). Incubation of either agonist alone (100 μM) with no tissue, resulted in no production of iP (data not shown). Further, time-dependent controls (Figure 7) showed that the tissue alone did not produce any iP over the course of the experiments. In contrast to the findings with ATP and 2-MeSATP, incubation of tissues with α,β-meATP led to little increase in iP in the 2 h period (Figure 7a).

However, in the absence of divalent cations and with the addition of 1 mM EDTA, there was little or no increase in iP with ATP or 2-MeSATP (both 100 μM) so that the levels of iP were not different from control preparations that had received no agonist ($n = 4$; Figure 7b).

Discussion

Cell surface receptors for ATP, termed P_2-purinoceptors have been subdivided into P_{2X} and P_{2Y} subtypes largely on the basis of rank orders of agonist potencies (Burnstock & Ken-

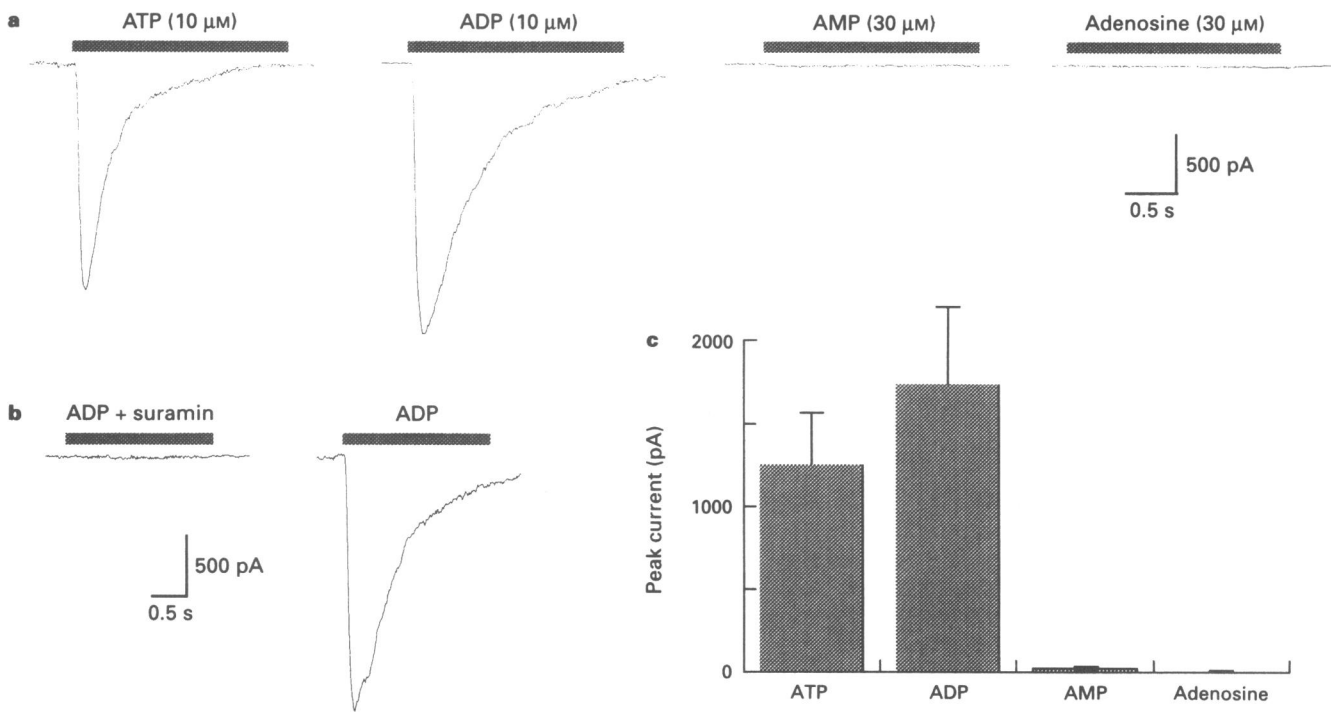

Figure 5 Actions of ADP, AMP and adenosine on single vas deferens smooth muscle cells. (a) Current recordings from different cells showing that ADP and ATP but not AMP and adenosine evoked inward currents. (b) Application of ADP (10 μM) in the presence of suramin (100 μM) evoked no inward current while subsequent application of ADP after suramine washout evoked a large inward current in the same cell (one of two such observations shown). (c) Summary of all experiments shown in (a) where values are mean ± s.e.mean of 4–5 cells.

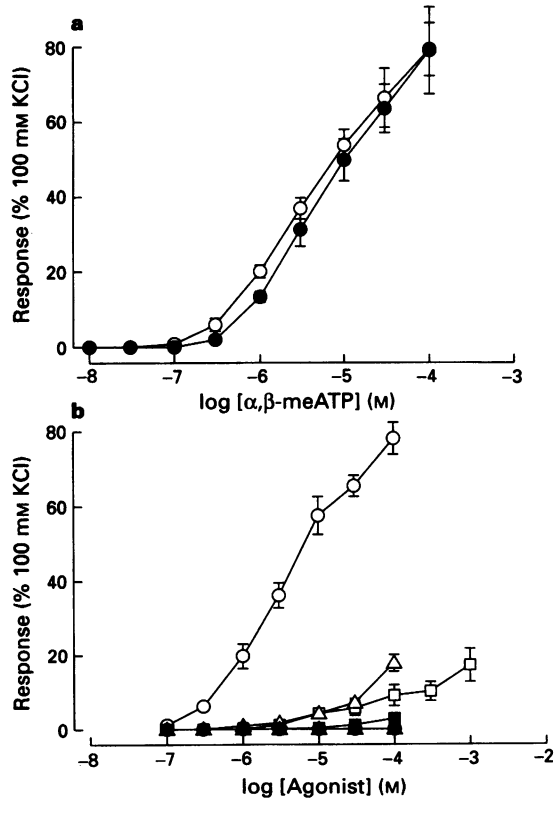

Figure 6 Contractile action of ATP and its structural analogues in rat isolated vas deferens. (a) Time-dependent controls, showing the first (○) and second control concentration-effect curves (●) to α,β-meATP determined with a 1 h washout between curves. Note curves to α,β-meATP were reproducible over the course of experimentation. (b) Second concentration-effect curves to ATP (□), 2-MeSATP (△), α,β-meATP (○), ADP (■), AMP (▲) and adenosine (●). Data shown are mean ± s.e.mean from at least four rats.

nedy, 1985). The structural analogue of ATP, α,β-meATP has been considered a potent and selective agonist at P_{2X}-purinoceptors and 2-MeSATP a potent agonist at P_{2Y}-purinoceptors (Burnstock & Kennedy, 1985). Much of the data supporting the original classification of the P_{2X}-purinoceptors derived from studies carried out in whole isolated preparations. The possible contribution of differential agonist breakdown to the rank orders of agonist potencies identified has been considered by a number of investigators. Nevertheless the caveat seems to have been largely disregarded and data with these agonists has been widely used in the characterization of P_2-purinoceptors using functional studies in a variety of tissues (Burnstock, 1990; Kennedy, 1990; Abbrachio & Burnstock, 1994; Abbrachio *et al.*, 1993; Fredholm *et al.*, 1994).

We have previously suggested that the low potency of ATP and 2-MeSATP in rat and guinea-pig vas deferens could be due to agonist breakdown or removal thus underestimating their true potency at P_{2X}-purinoceptors (Khakh *et al.*, 1994). We have now compared the agonist actions of ATP and a number of its structural analogues in single smooth muscle cells of rat vas deferens studied by single cell voltage clamp with those in isolated preparations of rat vas deferens measuring contraction. We have shown that, in the single cell studies, metabolically unstable analogues are equal in potency to the more stable ones, whereas in the isolated whole tissue preparations they are at least 10 to 30 times less potent. We suggest that the current approach to the characterization of P_{2X}-purinoceptors, based on relative agonist potencies, is inappropriate (Burnstock & Kennedy, 1985; Burnstock, 1990; Kennedy, 1990; Abbrachio *et al.*, 1993; Abbrachio & Burnstock, 1994; Fredholm *et al.*, 1994), since it relies on data where the potency of the metabolically unstable agonists is underestimated. We propose that ATP and 2-MeSATP must be considered as potent agonists at smooth muscle P_{2X}-purinoceptors, the archetypal and selective P_{2X}-purinoceptor agonist, α,β-meATP, being no more potent. Furthermore there is now evidence that P_{2X}-

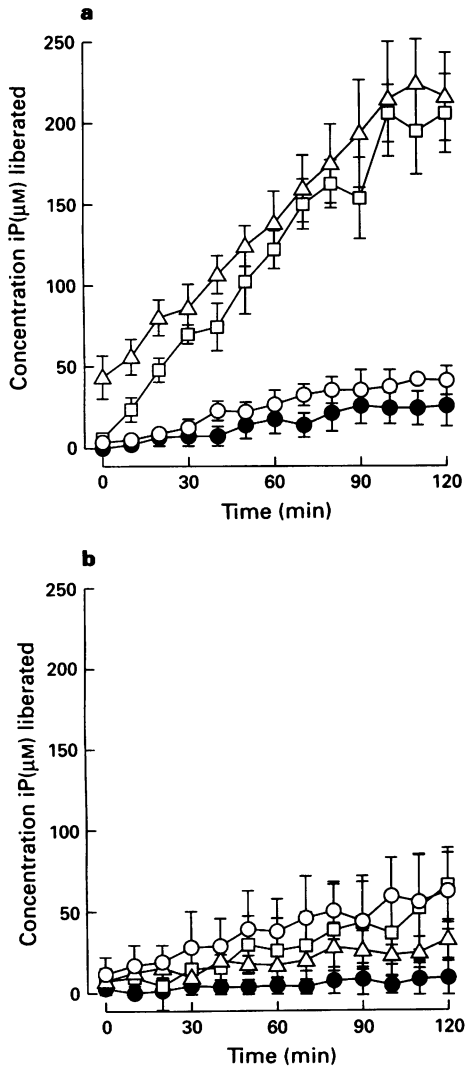

Figure 7 Inorganic phosphate production from purine nucleotides in isolated vas deferens. (a) Graph of increase in iP with time following incubation with 100 μM ATP (□), 2-MeSATP (△) and α,β-meATP (○) as compared to control (●) in bisected vas deferens. Note incubation with ATP or 2-MeSATP both resulted in significant increases in iP whereas α,β-meATP did not. (b) As in (a) but buffer contains 1 mM EDTA and no Ca^{2+} or Mg^{2+}. Note that little iP is produced following incubation with ATP, 2-MeSATP or α,β-meATP under these conditions. Data shown are mean ± s.e.mean from 4–8 rats.

purinoceptors in neurones are different from those in smooth muscle but similar problems concerning the use of purine nucleotide agonists in their characterization would seem to apply (see Trezise *et al.*, 1994b; 1995; Khakh *et al.*, 1995).

Single cell studies

Agonists were applied to the myocytes using the fast flow U tube method (Fenwick *et al.*, 1992). ATP, 2-MeSATP and α,β-meATP all evoked inward currents in every cell tested. The agonists evoked inward currents with rise times of less than 60 ms at 10 μM each agonist, which is consistent with a direct action at a ligand-gated ion channel (Inoue & Brading, 1990). Rapid rise times to peak effect followed by waning of the response during the continued presence of agonist were a feature of all agonists tested in this study, although α,β-meATP evoked inward currents with a slower rise time and decay constant than either ATP or 2-MeSATP. The slower rise time for the α,β-meATP-evoked current relative to ATP and 2-MeSATP is consistent with other studies on smooth muscle (Evans & Kennedy, 1994) and autonomic neurones (Khakh *et al.*, 1995). These differences in kinetics may reflect either a slower rate of association with the receptor or a lower intrinsic efficacy of α,β-meATP for P_{2X}-purinoceptors. A difference between decay constants for α,β-meATP, ATP and 2-MeSATP has also been reported in dissociated rat tail artery smooth muscle cells (Evans & Kennedy, 1994) and this may reflect differing affinities of the agonists for the open and desensitized states of the channel.

Responses mediated by neuronal P_{2X}-purinoceptors in autonomic ganglia exhibit little if any wane during agonist application for up to 20 s applications (Khakh *et al.*, 1995). This is in contrast to the profound wane seen in the present study for smooth muscle P_{2X}-purinoceptors (see Figures 1–3). In addition responses to each of the agonists were tachyphylactic when administered up to 6 min apart (Figure 1). This finding is consistent with that of Evans & Kennedy (1994) and Nakazawa & Matsuki (1987) but different from studies of Friel (1988) and Inoue & Brading (1990). However, the gene encoding for the P_{2X}-purinoceptor from rat vas deferens has recently been cloned and similar patterns of wane and tachyphylaxis were observed for the responses mediated by the recombinant P_{2X}-purinoceptors expressed and studied in oocytes and mammalian cells (Valera *et al.*, 1994). Thus, the tachyphylaxis associated with the smooth muscle P_{2X}-purinoceptors seen in our study probably results from the structure of the receptor-channel rather than the experimental procedure.

The most discordant observation of Friel (1988) was that α,β-meATP was not an agonist in smooth muscle cells, whereas we found that it was equal in potency to ATP and 2-MeSATP. However our findings are broadly consistent with published data in rat tail artery (Evans & Kennedy, 1994), bladder (Inoue & Brading, 1990) and for the recombinant P_{2X}-purinoceptor expressed in HEK cells (Valera *et al.*, 1994). In this study, full concentration-effect curves could not be obtained to agonists since at the highest concentrations tested, the rise times for the responses were approaching the rise times for adequate solution exchange, thus higher concentrations of agonist would have underestimated the peak amplitude. However, from comparison of threshold concentrations and peak amplitude responses it would appear that there was little or no difference in potencies between the three agonists.

Two pertinent questions arise from these findings. First, do all the agonists act at a common receptor on the smooth muscle cell? We consider that this is likely to be the case since all the agonists were antagonized by the P_2-purinoceptor antagonist, suramin (Dunn & Blakeley, 1988) and also the agonists cross-desensitized each other, indicative of a common site of action (unpublished observation; see also Evans & Kennedy, 1994). Secondly, is the ATP-evoked inward current responsible for smooth muscle depolarization and subsequent contraction in isolated whole tissue preparations? Although we have not investigated this in the present study we feel that this is a reasonable supposition, because in bladder muscle cells the depolarization induced by ATP resembles closely the profile of the e.j.p. measured from isolated tissues, supporting the contention that the mechanisms of the responses measured in single cells underlies that of the contraction of isolated preparations (Inoue & Brading, 1990).

Measurement of contractile activity

The finding that ATP is more potent that ADP, AMP or adenosine in contracting the bisected vas deferens preparation is consistent with previous findings (Taylor *et al.*, 1982) and suggests the involvement of a P_2-purinoceptor (Burnstock, 1978). Furthermore the high potency of α,β-meATP relative to ATP and 2-MeSATP suggests the involvement of a P_{2X}-purinoceptor (Abbrachio & Burnstock, 1994). Based on the experiments in this study on rat whole tissue vas deferens

a rank order of agonist potencies of α,β-meATP>>ATP = 2-MeSATP was found which is that expected of a P_{2X}-purinoceptor (Burnstock & Kennedy, 1985).

However, the original classification of P_{2X}-purinoceptors (Burnstock & Kennedy, 1985) does not addresss the shallow concentration-effect curve or low potency of ATP as an agonist in whole tissue preparations such as the rat vas deferens. One possible explanation for the low potency of ATP and 2-MeSATP is that in isolated tissue preparations they are metabolised rapidly thereby underestimating their true potency at P_{2X}-purinoceptors. In contrast, in single cells significant purine metabolism is unlikely to occur during rapid agonist applications, where currents are measured over milliseconds without diffusion gradients. In order to investigate this explanation further we have measured the production of iP, expected to result from the metabolism of purine nucleotides in whole tissue studies.

Measurement of inorganic phosphate

Incubation of rat isolated vas deferens in the presence of either 100 μM ATP or 2-MeSATP produced a large increase in iP over a 2 h period, which amounted to approximately 200 μM iP from 100 μM nucleotide agonist. In control preparations which received no agonist, no iP was detectable. It seems reasonable to assume that the measurement of iP production underestimates the true rate of metabolism of purine nucleotides in the vicinity of the receptor. Thus the quantity of purine nucleotide in the bathing medium is far in excess of physiological levels and the metabolising enzymes may be closely associated with the receptor in the synapse as is the case for acetylcholinesterase and the nicotinic receptor (Zimmerman *et al.*, 1994). Hence iP levels can only be used as a measure of relative stability of analogues and not as a measure of rate of metabolism in the immediate environment of the receptor. α,β-meATP, unlike ATP and 2-MeSATP, produced no increase in iP for up to 2 h, indicating that it is metabolically stable. These findings indicating that ATP and 2-MeSATP are both metabolically unstable and that α,β-meATP is in contrast stable to degradation are in good agreement with previous data (Welford *et al.*, 1986; 1987).

Complete removal of Ca^{2+} and Mg^{2+} and addition of 1 mM EDTA prevented the increase in iP from ATP and 2-MeSATP; we assume this reflects the Ca^{2+}- and Mg^{2+}-dependency of the enzyme responsible for metabolism. It has also been shown that the enyzmes responsible for the breakdown of ATP in oocytes are sensitive to complete removal of Ca^{2+} and Mg^{2+} (Ziganshin *et al.*, 1995). Further, in isolated vagus nerve evidence has been provided, using indirect methods, that metabolism of ATP is dependent on submicromolar concentrations of Ca^{2+} and Mg^{2+}, such that inhibition of metabolism can only be achieved by using 1 mM EDTA (Trezise *et al.*, 1994a,b). Thus our findings are in agreement with those of others (Ziganshin *et al.*, 1995; Trezise *et al.*, 1994a,b) which suggest that the enzyme(s) involved belongs to a family of Ca^{2+} and Mg^{2+}-dependent ectonucleotidases.

Summary

Caveats concerning the use of agonists in drug receptor classification are well known (Kenakin, 1993). In the present study we have demonstrated in rat vas deferens, one of the preparations in which the role of ATP as a co-transmitter was first established and purinoceptors first classified (see von Kügelgen & Starke, 1991; Burnstock & Kennedy, 1985), that an agonist-based classification of P_{2X}-purinoceptors is misleading because of problems associated with underestimating the potency of metabolisable analogues such as ATP and 2-MeSATP. We suggest, on the basis of our single cell studies, that ATP and 2-MeSATP must be considered as potent agonists for the P_{2X}-purinoceptors in smooth muscle, equal in potency to the archetypal agonist, α,β-meATP.

Unequivocal characterization of P_{2X}-purinoceptors in functional studies can only be achieved on the basis of affinity estimates with potent competitive antagonists. However it is reassuring to note that the agonist profile of the recombinant P_{2X}-purinoceptor from rat vas deferens (Valera *et al.*, 1994) is remarkably similar to that which we suggest as truly characteristic of P_{2X}-purinoceptors in vas deferens smooth muscle.

The authors appreciate the useful and informative discussions with Drs Richard Evans, Wasyl Fenuik, Ian Kennedy, Anton Michel, Alan North and Derek Trezise.

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(Received November 3, 1994
Revised January 5, 1995
Accepted January 24, 1995)