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Pharmacological properties of $P2X_3$-receptors present in neurones of the rat dorsal root ganglia

[1]M.G. Rae, E.G. Rowan & [2]C. Kennedy

Department of Physiology and Pharmacology, University of Strathclyde, Royal College, 204 George St, Glasgow, G1 1XW

1 The electrophysiological actions of several agonists which may differentiate between $P2X_1$- and $P2X_3$-receptors were studied under concentration and voltage-clamp conditions in dissociated neurones of 1–4 day old rat dorsal root ganglia.

2 β,γ-Methylene-D-ATP (β,γ-me-D-ATP) (1–300 μM), diadenosine 5′,5‴-P^1,P^5-pentaphosphate (AP5A) (100 nM–300 μM), diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AP4A) (300 nM–300 μM) and uridine 5′-triphosphate (UTP) (1 μM–1 mM) all activated concentration-dependent inward currents with a latency to onset of a few ms.

3 The concentration-response curves for β,γ-me-D-ATP and AP5A and ATP had similar maximum values, while that for AP4A had a lower maximum. The concentration-response curve to UTP was shallow and did not reach a maximum. β,γ-Methylene-L-ATP was virtually inactive. The rank order of agonist potency was ATP > AP5A ≈ AP4A > β,γ-me-D-ATP > UTP > > β,γ-methylene-L-ATP.

4 The inward currents were inhibited by the P2-receptor antagonists suramin (100 μM) and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (10 μM). PPADS also inhibited responses to ATP (800 nM) and α,β-methylene ATP (2 μM) in a concentration-dependent manner.

5 This study shows that β,γ-me-D-ATP, AP5A, AP4A and UTP all act via a suramin- and PPADS-sensitive P2X-receptor to evoke rapid, transient inward currents in dissociated neurones of rat dorsal root ganglia. The very low activity of β,γ-methylene-L-ATP suggests that the agonists were acting at the $P2X_3$-subtype to produce these effects.

Keywords: Rat dorsal root ganglia; sensory neurones; P2X-receptor; ATP; β,γ-methylene-L-ATP

Introduction

P2X-receptors are ligand-gated cation channels which mediate the excitatory actions of extracellular adenosine 5′-triphosphate (ATP) in many tissues, including sensory neurones in dorsal root ganglia (Bean, 1990; Bouvier *et al*., 1991; Robertson *et al*., 1996). The presence of P2X-receptors on sensory neurones may underlie the ability of exogenous ATP to cause pain in human (Bleehen *et al*., 1976; Bleehen & Keele, 1977) and animal (Bland-Ward & Humphrey, 1997; Sawynok & Reid, 1997) models and to excite cutaneous (Bleehen, 1978; Rea & Wallis, 1992; Trezise & Humphrey, 1996) and visceral (Collier *et al*., 1966; Pelleg & Hurt, 1996) afferent nerves. It is also consistent with data showing that P2X-antagonists are analgesic in man, rats and mice (Ho *et al*., 1992; Driessen *et al*., 1994). Consequently, it has been proposed that ATP released in the vicinity of sensory nerve endings may be a physiological noxious stimulus (see Kennedy & Leff, 1995; Burnstock, 1996; Burnstock & Wood, 1996).

Seven subtypes of P2X-receptor have been identified in expression cloning studies (see Buell *et al*., 1996; North, 1996 for reviews). The basic biophysical and pharmacological properties of native P2X-receptors present in dissociated sensory neurones of the rat dorsal root ganglia indicate the presence of $P2X_1$- and/or $P2X_3$-receptors, as the P2X-receptor agonists ATP, 2-methylthioATP and α,β-methyleneATP (α,β-meATP) all evoked rapid, transient inward currents, which were inhibited by the P2X-receptor antagonist suramin (Robertson *et al*., 1996). mRNA for the $P2X_1$-subtype (Lewis *et al*., 1995; Collo *et al*., 1996; but see Grahames *et al*., 1996), as well as the $P2X_3$-subtype (Chen *et al*., 1995; Lewis *et al*., 1995; Collo *et al*., 1996) has been found in rat dorsal root ganglia. Thus, the response to the P2X-receptor agonists could in theory be explained by the presence of $P2X_1$-receptors alone, by $P2X_3$-receptors alone or by a mixture of the two.

The agonists used thus far in these studies are non-selective between $P2X_1$- and $P2X_3$-receptors. In order to differentiate between the two subtypes, selective ligands are required and several other compounds are possible candidates. β,γ-Methylene-L-ATP (β,γ-me-L-ATP) is an agonist at $P2X_1$-receptors in smooth muscle, but not at P2X-receptors in neurones, whereas its stereoisomer β,γ-methylene-D-ATP (β,γ-me-D-ATP) is active at both sites (Trezise *et al*., 1995). Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (AP4A) and diadenosine 5′,5‴-P^1,P^5-pentaphosphate (AP5A) are also active at $P2X_1$-receptors (Evans *et al*., 1995; Westfall *et al*., 1997), but their effect at $P2X_3$-receptors is unknown. Finally, uridine 5′-triphosphate (UTP) has been shown to activate the cloned $P2X_3$-receptor (Chen *et al*., 1995), but not the cloned $P2X_1$-receptor (Valera *et al*., 1994).

The aim of this study was to investigate which P2X-receptors are functionally expressed in neurones of the rat dorsal root ganglia by use of the agonists discussed above. The effects of the P2X-receptor antagonists suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) were also determined. A preliminary account of these results has been published (Rae *et al*., 1996).

Methods

Single cell isolation

Neurones of rat dorsal root ganglia were isolated by means of a simpler version of the protocol described previously

[1]Department of Physiology, University of Nevada School of Medicine, Reno, Nevada 89557, U.S.A.
[2]Author for correspondence.

(Robertson *et al*., 1996). Rat pups (1–4 days old) were killed by cervical dislocation then decapitation. The ganglia were dissected out and incubated for 15 min at 37°C in 2 ml of a modified Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution containing 0.25% trypsin. The ganglia were then transferred to 2 ml Ca^{2+}/Mg^{2+}-free Hank's solution containing collagenase Type 1 (1.25 mg ml^{-1}) for a further 5 min. Very mild trituration was then performed with a 1 ml Gilson pipette, 3 ml L-15 solution was then added and the cells centrifuged at 75 g for 5 min. The cells were re-suspended in 3 ml L-15 solution, centrifuged at 75 *g* for 5 min and finally re-suspended in a modified L-15 medium containing 10% foetal calf serum, $NaHCO_3$ (26 mM), glucose (30 mM) and nerve growth factor (100 ng ml^{-1}). The cells were plated onto glass coverslips (Fisons) coated with poly-L-lysine (0.1 mg ml^{-1}, mol wgt = 70,000–150,000) and grown at 37°C in a 100% humidified 95% air/5% CO_2 atmosphere. Cells were used within 2 days.

Electrophysiological recordings

Cells were superfused (2 ml min^{-1}) at room temperature with a solution of the following composition (mM): NaCl 140, KCl 5, Na_2HPO_4 0.06, glucose 10, HEPES 10, $MgCl_2$ 1.2, $CaCl_2$ 2.5, titrated to pH 7.3 with NaOH and osmolarity 310 mOsm. Whole cell currents were recorded in cells of 20–40 μm diameter by use of the patch clamp technique with an Axopatch 1D amplifier. The resistance of the pipettes was 2–5 MΩ when filled with a solution containing (mM): KCl 145, $MgCl_2$ 2, HEPES 10, EGTA 5, Na_2ATP 2, Na_2GTP 0.1, tritrated to pH 7.03 with KOH and osmolarity 300 mOsm. Pipettes were coated with Sigmacoat to reduce capacitance artefacts. The liquid junction potential was subtracted by use of the d.c. offset on the amplifier. In most cells series resistance compensation was at least 80%. In all experiments the membrane potential was clamped at −60 mV. Data were collected and analysed on an IBM-compatible PC using WCP software (Dr J Dempster, Strathclyde University) with a National Instruments Lab PC plus interface at a sampling frequency of 1 KHz.

Application of drugs

Agonists were applied for 500 ms at 10 min intervals by use of a solenoid valve-controlled U-tube application system placed 200 μm from the cell (see Robertson *et al*., 1996). The equilibration time was generally less than 10 ms. Routinely, at the start of each recording a 500 ms pulse of the extracellular bathing solution was applied to a cell via the U-tube and any cells displaying a change in holding current (i.e. mechanical artefacts) were discarded. The first application of agonist was applied to the cell 5 min after whole cell mode was achieved, in order to standardize any time-dependent effects of cell dialysis. Only one cell per coverslip was tested for responses to the drugs and in most cases only a single concentration of a given agonist was applied. Antagonists, applied in the superfusate, were allowed to equilibrate with the cells for 5 min.

Statistics

Data are expressed throughout as the mean ± s.e.mean and were compared by one way analysis of variance and Tukey's comparison, with $P<0.05$ considered to be statistically significant. Concentration-response curves were fitted to the data by logistic (Hill equation), non-linear regression analysis (Fig. P., Biosoft, Cambridge, U.K.).

Drugs and solutions

Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution, L-15 (Leibowitz) medium, collagenase Type 1, trypsin/EDTA, $NaHCO_3$ 7.5% solution, glucose 45% solution, foetal calf serum, nerve growth factor and poly-L-lysine were obtained from Sigma. Stock solutions of ATP (sodium salt), α,β-meATP (lithium salt), uridine 5′-triphosphate (UTP) (sodium salt), AP_4A (ammonium salt), AP_5A (sodium salt), (all Sigma), β,γ-me-D-ATP, β,γ-me-L-ATP (Research Biochemicals Inc), PPADS (Tocris Cookson, U.K.) and suramin (Bayer, U.K.) were dissolved in deionized water and stored frozen. All drugs were then diluted in extracellular bathing solution.

UTP provided by Sigma was assayed by h.p.l.c. and found to be 86–95% pure. ATP was not present and no other nucleotide was identified. When the UTP was purified it was still as effective at evoking the inward currents (not shown).

Results

Responses to agonists

Isolated neurones of the rat dorsal root ganglia had a resting membrane potential of −54.9 ± 0.6 mV and cell capacitance of 17.5 ± 0.4 pF ($n=513$); 486/513 neurones (95%) were responsive to the P2X-agonists tested. For each agonist the inward currents had a time to onset of a few ms, consistent with activation of a ligand-gated channel. Also, similar to ATP, as the agonist concentration increased, so the currents reached a peak and decayed more rapidly (not shown). In initial experiments, the potency of each agonist was determined and compared with ATP.

β,γ-me-D-ATP (1–300 μM) evoked rapidly developing inward currents in a concentration-dependent manner, but was less potent than ATP (Figure 1, ATP-EC_{50} = 719 nM, Hill slope = 1.47; β,γ-me-D-ATP-EC_{50} = 13.1 μM, Hill slope = 1.76). Whilst the mean maximum current evoked by β,γ-me-D-ATP was about two-thirds of that evoked by ATP, they were not significantly different. In contrast, β,γ-me-D-ATP was almost inactive, only evoking small inward currents at 100 μM (132 ± 22 pA, $n=14$) and 300 μM (167 ± 27 pA, $n=15$) (Figure 1b). The actions of β,γ-me-L-ATP were not studied further.

AP5A (100 nM–300 μM) and AP4A (300 nM–300 μM) also evoked rapidly developing inward currents in a concentration-dependent manner with a similar potency, but both were less potent than ATP (Figure 2). The EC_{50} and Hill slope values were 3.2 μM and 0.98 for AP5A and 5.6 μM and 1.13 for AP4A. Whereas the maximum currents evoked by AP5A and ATP were not significantly different, the maximum current to AP4A was significantly smaller ($P<0.05$).

Finally, UTP (1 μM–1 mM) evoked inward currents similar to the other agonists and was much less potent than ATP (not shown). However, the concentration-response curve was shallow and did not reach a maximum, so an EC_{50} value could not be calculated. Thus, the agonist potency order in these cells was ATP > AP5A ≈ AP4A > β,γ-me-D-ATP > UTP > > β,γ-me-L-ATP.

Effects of suramin and PPADS

We have previously shown that the P2-receptor antagonist suramin (100 μM) inhibited inward currents evoked by ATP, 2-

methylthioATP, α,β-meATP and UTP in these cells (Robertson *et al.*, 1996). Suramin (100 μM), applied for 5 min in the superfusate, also inhibited the inward currents evoked by β,γ-me-D-ATP (30 μM) by 95±4%, AP5A (10 μM) by 96±2% and AP4A (30 μM) by 75±1% ($n=3$ each). Reversal of the inhibitory action of suramin developed slowly, even though its washout was rapid (not shown). Thus, in common with ATP, each of the agonists is acting at a suramin-sensitive site.

PPADS (10 μM), applied for 5 min in the superfusate, abolished the currents evoked by ATP (800 nM, $n=3$, Figure 3), α,β-meATP (2 μM, $n=4$, Figure 3), β,γ-me-D-ATP (30 μM, $n=4$), AP4A (30 μM, $n=3$), AP5A (10 μM, $n=3$) and UTP (50 μM, $n=6$). Similar to suramin, reversal of the inhibitory action of PPADS developed slowly, even though its washout was rapid (not shown).

The concentration of PPADS (10 μM) used was clearly supramaximal. When ATP (800 nM) and α,β-meATP (2 μM) were used as the test agonists, PPADS (100 nM and 1 μM) inhibited, but did not abolish the inward currents (Figure 3). The inhibition was concentration-dependent and approximately 50% inhibition was seen at 100 nM PPADS.

Discussion

In this study, several P2X-receptor agonists evoked suramin- and PPADS-sensitive inward currents in dissociated sensory neurones of neonatal rat dorsal root ganglia. The currents activated within a few ms, reached a peak rapidly and desensitized in the continued presence of agonist. In an earlier

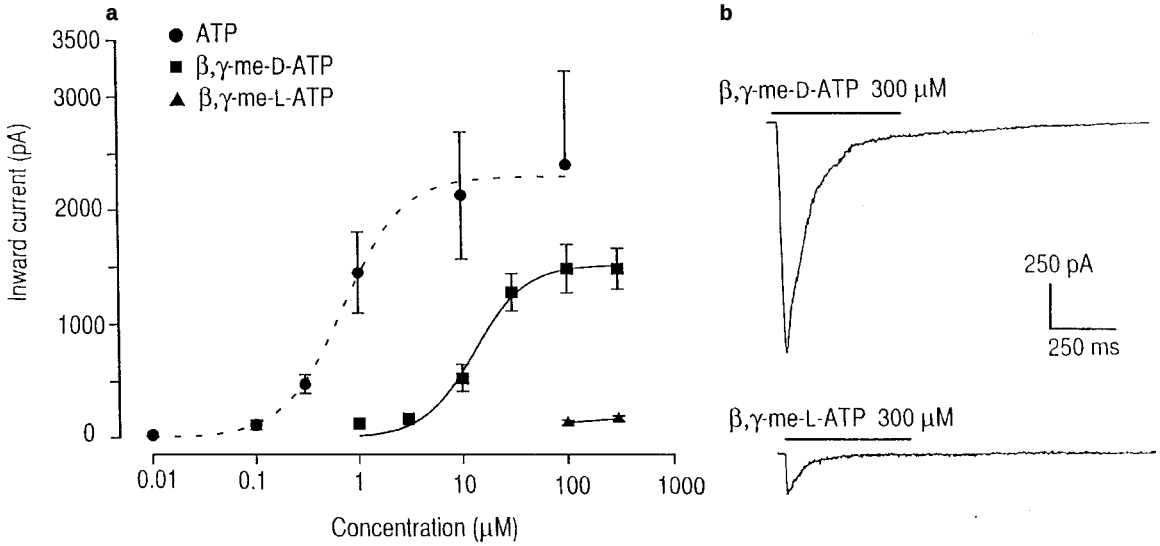

Figure 1 Fast inward currents evoked by β,γ-me-D-ATP and β,γ-me-L-ATP in acutely dissociated neurones of the rat dorsal root ganglia. (a) Mean peak inward current amplitude is plotted against log concentration of β,γ-me-D-ATP and β,γ-me-L-ATP. The log concentration-response curve for ATP from Robertson *et al.* (1996) is also plotted to allow comparison of agonist potency. Vertical lines show s.e.mean, ($n=7-28$). (b) Traces show inward currents evoked by β,γ-me-D-ATP (upper) and β,γ-me-L-ATP (lower) (300 μM) when applied rapidly for 500 ms, as indicated by the solid bars.

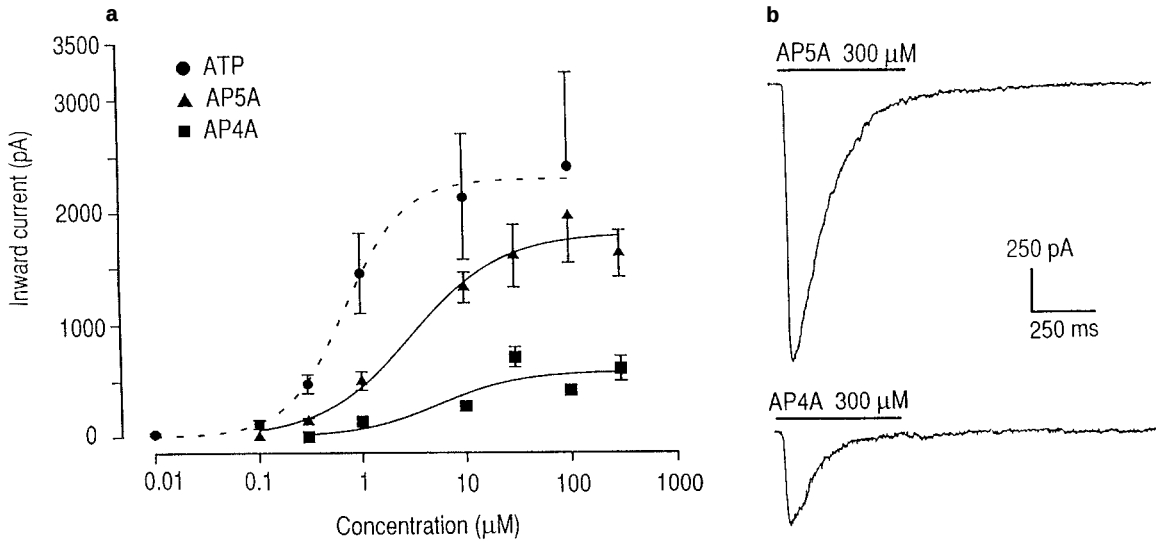

Figure 2 Fast inward currents evoked by AP4A and AP5A in acutely dissociated neurones of the rat dorsal root ganglia. (a) Mean peak inward current amplitude is plotted against log concentration of AP4A and AP5A. The log concentration-response curve for ATP from Robertson *et al.* (1996) is also plotted to allow comparison of agonist potency. Vertical lines show s.e.mean ($n=5-31$). (b) Trace show inward currents evoked by AP5A (upper) and AP4A (lower) (300 μM) when applied rapidly for 500 ms, as indicated by the solid bars.

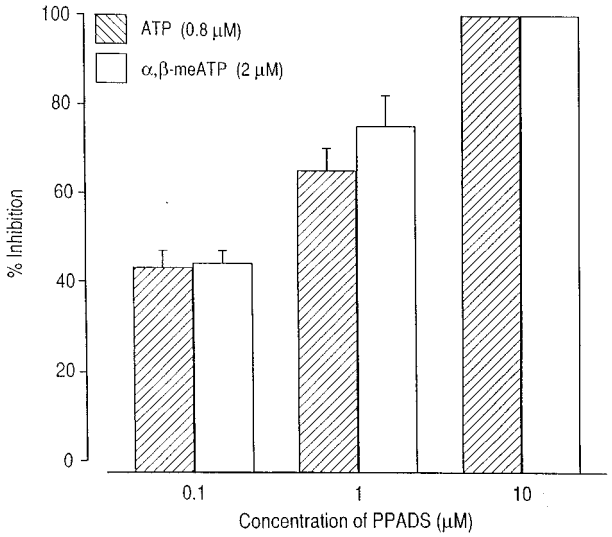

Figure 3 PPADS inhibits inward currents evoked by ATP and α,β-meATP in acutely dissociated neurones of the rat dorsal root ganglia. Mean inhibition by PPADS of peak amplitude of inward currents evoked by ATP and α,β-meATP is plotted against log concentration of PPADS. Vertical lines show s.e.mean ($n=4-15$).

study, ATP and α,β-meATP evoked similar currents (Robertson *et al*., 1996). The rapid desensitization and potent activity of α,β-meATP indicated activation of $P2X_1$- and/or $P2X_3$-receptors. The present results eliminate $P2X_1$-receptors as the site of action and suggest that the currents were mediated by $P2X_3$-receptors only.

The strongest evidence for functional expression of $P2X_3$- rather than $P2X_1$-receptors, is that in this study β,γ-me-D-ATP evoked inward currents at μM concentrations, but β,γ-me-L-ATP was almost inactive. β,γ-me-L-ATP is also inactive at P2X-receptors in sensory neurones of the rat nodose ganglia (Trezise *et al*., 1995), but is a potent agonist at cloned (Evans *et al*., 1995) and native $P2X_1$-receptors in smooth muscle (Cusack & Hourani, 1984; Hourani *et al*., 1986; Trezise *et al*., 1995; von Kügelgen *et al*., 1995) and displaces radioligand binding at the cloned $P2X_1$-receptor (Michel *et al*., 1996). β,γ-me-D-ATP is also an agonist at native and cloned $P2X_1$-receptors, but is less potent than its stereoisomer (Cusack & Hourani, 1984; Hourani *et al*., 1986; Evans *et al*., 1995). Thus, the inactivity of β,γ-me-L-ATP and the greater potency of β,γ-D-ATP, seen in the present study, indicate that $P2X_1$-receptors are not present in sensory neurones of the neonatal rat dorsal root ganglia. In turn, this suggests that the $P2X_3$-receptor is functionally expressed in these cells, as of the seven P2X-subtypes, only the $P2X_3$-receptor fits the pharmacological and biophysical profile of the responses seen.

This conclusion is consistent with data showing that mRNA for the $P2X_3$-subtype is only found at high levels in sensory neurones, including rat dorsal root ganglia (Chen *et al*., 1995; Lewis *et al*., 1995; Collo *et al*., 1996). Pretreatment with capsaicin substantially reduces the level of $P2X_3$-mRNA (Chen *et al*., 1995), suggesting that $P2X_3$-receptors are selectively expressed in C-fibre afferents, which are mainly nociceptive. The same conclusion has been reached from studies on sensory neurones of rat tooth pulp, all of which are nociceptive (Cook *et al*., 1997).

The other compounds used in the present study were active at the $P2X_3$ receptor, but are unlikely to be useful in identifying P2X-receptor subtypes. UTP activated inward currents similar to ATP, but with much lower potency. UTP was studied as it activates the $P2X_3$-receptor cloned from sensory neurones (Chen *et al*., 1995), but not the $P2X_1$-receptor cloned from visceral smooth muscle (Valera *et al*., 1994), nor the native $P2X_1$-receptor in smooth muscle of the rat vas deferens (Friel, 1988). Thus, it was anticipated that UTP might differentiate between $P2X_1$- and $P2X_3$-receptors, albeit with low potency. However, in a parallel study (McLaren *et al*., 1997) we found that UTP activates the native $P2X_1$-receptor in vascular smooth muscle cells at the same concentrations as used here. Thus, UTP does not discriminate between $P2X_1$- and $P2X_3$-receptors.

In this study, AP4A and AP5A activated inward currents with a similar time-course to ATP. This is the first time activity of the latter has been demonstrated at $P2X_3$-receptors. The maximum response to AP4A was smaller than those to ATP and AP5A, indicating that AP4A is a partial agonist at this site, as it is in rat nodose ganglion (Krishtal *et al*., 1988). Its low efficacy may make it a useful starting point for the development of a $P2X_3$-antagonist. The actions of AP4A and AP5A at the cloned $P2X_3$-receptor have not been demonstrated, but AP5A is a partial agonist at the cloned $P2X_1$-receptor (Evans *et al*., 1995) and displaces radioligand binding at this site (Michel *et al*., 1996). Both compounds are also agonists at native $P2X_1$-receptors in the guinea-pig vas deferens (Westfall *et al*., 1997).

The P2X-antagonists suramin and PPADS also do not discriminate between $P2X_1$- and $P2X_3$-receptors. In this and our previous study (Robertson *et al*., 1996), suramin and PPADS abolished or almost totally inhibited responses to each of the P2X-agonists tested. Both are also antagonists at the cloned $P2X_1$- (Valera *et al*., 1994; Evans *et al*., 1995) and $P2X_3$-receptors (Chen *et al*., 1995; Lewis *et al*., 1995). Both antagonists also inhibit the binding of a specific radiolabelled ligand to either cloned receptor (Michel *et al*., 1996; Miller *et al*., 1996).

In conclusion, the ability of α,β-meATP to activate rapid, transient inward currents, together with the lack of ability of β,γ-me-L-ATP to do likewise, indicates that the P2X-receptor present in sensory neurones of neonatal rat dorsal root ganglia is most likely the $P2X_3$-subtype. The data also suggest that β,γ-me-L-ATP may be of some use in discriminating between $P2X_1$- and $P2X_3$-receptors.

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