British Journal of Pharmacology (2000) 129, 1063–1066
www.nature.com/bjp

SPECIAL REPORT

The $P2Y_1$ receptor closes the N-type Ca^{2+} channel in neurones, with both adenosine triphosphates and diphosphates as potent agonists

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The rat $P2Y_1$ nucleotide receptor, the P2Y subtype abundant in the brain, was heterologously expressed in rat superior cervical ganglion neurones by micro-injection of the receptor cRNA or cDNA. ADP inhibited the N-type Ca^{2+} current by 64%, with EC_{50} 8.2 nM, an action blocked competitively by the $P2Y_1$ receptor antagonist adenosine 3′, 5′-*bis*-phosphate (K_i 0.7 μM). 2-Methylthio-ADP inhibited the Ca^{2+} current likewise, but with EC_{50} 0.57 nM, giving the highest potency reported therewith for $P2Y_1$. Significantly, ATP and 2-methylthio-ATP were also agonists, the latter again at a very high potency (EC_{50} 2.5 nM). We propose that this neuronal receptor, when present in brain at a high density as at synapses, can respond to very low concentrations of ATP and ADP as agonists, and that this would result in inhibition of N-type Ca^{2+} currents and hence can reduce transmitter release or increase neuronal excitability.
British Journal of Pharmacology (2000) **129,** 1063–1066

Keywords: Nucleotide receptors; $P2Y_1$ receptor; ATP; Ca^{2+} channel; transduction pathway

Abbreviations: GFP, Green Fluorescent Protein; 2-MeS, 2-methylthio; $P2Y_1$, $P2Y_1$ nucleotide receptor; SCG, superior cervical ganglion

Introduction The $P2Y_1$ receptor ($P2Y_1$) belongs to the P2Y family of the G-protein coupled nucleotide receptors (North & Barnard, 1997). Unlike other P2Y nucleotide receptors, $P2Y_1$ is expressed in high abundance in many brain regions (Barnard *et al*., 1997; Webb *et al*., 1998), as well as on some peripheral neurones. Its primary transduction mechanisms in neurones have not, however, been established. In heterologous expression in non-neuronal cells it stimulates the phosphoinositide signalling pathway and intracellular Ca^{2+} mobilization (Simon *et al*., 1995; Schachter *et al*., 1996; Hechler *et al*., 1998). Here we demonstrate a primary signalling action of the $P2Y_1$ receptor which can occur in neurones, the closure of a Ca^{2+} channel in the cell membrane. To reveal this step, we have employed a strategy which we recently found applicable in other cases (Filippov *et al*., 1998; 1999), whereby isolated single neurones of the superior cervical ganglion (SCG, where native P2Y receptors are negligible) are micro-injected with an mRNA or cDNA encoding the receptor, agonist is applied to the cell surface and any coupling to endogenous channels of the neurone is studied.

A parallel issue of current importance with $P2Y_1$ is the identity of the native agonists. When the $P2Y_1$ cDNA was originally cloned (Webb *et al*., 1993), both ADP and ATP (plus its more potent analogue, 2-methylthio-ATP (2-MeSATP)) were found to be agonists, using expression in *Xenopus* oocytes (Simon *et al*., 1995) and likewise for human $P2Y_1$ in a transfected cell line, monitoring inositol phosphate production (Schachter *et al*., 1996). Subsequently it was deduced, using the intracellular Ca^{2+} response in $P2Y_1$-expressing Jurkat cells, that the agonist action of ATP and 2-MeSATP was apparent only, due to the corresponding diphosphate agonists present or formed as an impurity therein (Leon *et al*., 1997) and that ATP is, in fact, a weak antagonist. ATP antagonism at $P2Y_1$ in a similar system was supported by Fagura *et al*. (1998), applying a theoretical correction for the effect of the ADP content (1%) found in commercial ATP samples. An apparently conclusive confirmation of this view has come when, in $P2Y_1$-expressing Jurkat cells, a rigorous exclusion from ATP samples of the presence or production of ADP abolished ATP agonism and led to it acting as an antagonist of the Ca^{2+} release response (Hechler *et al*., 1998).

Nevertheless, this question remains controversial, as Palmer *et al*. (1998) have reported that the $P2Y_1$-transfected 1321N1 astrocytoma cell line gave, in contrast, strong Ca^{2+} mobilization responses to purified ATP and 2-MeSATP as agonists, as well as to their diphosphates. The difference from the other findings was attributed to a much larger receptor reserve for $P2Y_1$ in the 1321N1 cells plus a lower intrinsic efficacy for ATP than for ADP. We address here both this question and the first-mentioned issue, for the mammalian $P2Y_1$ receptor acting in a neurone.

Methods Experimental procedures were identical to those described previously (Filippov *et al*., 1998; 1999) except where specified. The rat $P2Y_1$ cDNA (Webb *et al*., 1996) was inserted into the expression vector pcDNA-3 (Stratagene). This was used for the preparation of cRNA (as previously), used at 1.25 ng nl^{-1} (final concentration) in sterile water for injection into the cytoplasm of the single neurones on laminin-coated cover-slips, or for direct DNA injection into the nucleus at 150 ng μl^{-1} in sterile TE buffer (GIBCO). The Enhanced Green Fluorescent (mutant S65T) Protein (GFP) cDNA (Clontech) cloned into pcDNA-3, or its cRNA (prepared as above), was co-injected to identify the cells for recording. All cells used for controls expressed GFP alone. The neurones

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were then incubated at 37°C for 14–24 h, prior to external perfusion (20–25 ml min^{-1}, at 25°C) with Krebs-tetraethylammonium bathing solution (containing also 5 mM $BaCl_2$ as charge carrier). Agonist application began after a minimum time of 10–20 min after starting perfusion. The pharmacology of the current recorded was as in Filippov *et al*. (1999).

Nucleotides were dissolved in the bathing solution immediately before each application; the bath exchange time was 5 s. ADP, ATP and hexokinase were from Boehringer Mannheim, 2-MeSADP and 2-MeSATP from Research Biochemicals International and all other materials from Sigma (purest grades). ADP and 2-MeSADP were treated before use with hexokinase (10 U ml^{-1}) and glucose (0.1 M) in bathing solution for 2 h to remove all triphosphates (Nicholas *et al*., 1996). When a concentration-response curve for ADP was repeated with hexokinase (1 U ml^{-1})/glucose also present throughout in the perfusing solutions to ensure no later enzymatic conversion occurred, there was no significant change in the results obtained. ATP and 2-MeSATP were treated before use with creatine kinase (Type I, 20 U ml^{-1} and creatine phosphate (10 mM) for 90 min to convert all diphosphates to their triphosphates (Hechler *et al*., 1998).

Each concentration-response plot was determined progressively on a single cell, with 40 s total exposure at each point (less where stated) and the final curve for each agonist was fitted to the means of the data points combined from five cells, using the Hill equation, to give EC_{50} (mid-point location) and the Hill slope n_H. All values quoted are means ± s.e.mean. Student's *t*-test (unpaired) was applied to determine statistical significance ($P<0.05$).

Results In SCG neurones pre-injected with cRNA or cDNA encoding the rat $P2Y_1$ receptor, ADP inhibited the N-type Ca^{2+}-channel current, at concentrations down to 1 nM. This is as found with the $P2Y_6$ receptor expressed similarly (Filippov *et al*., 1998), but for UDP there, ADP then being inactive. In addition, the time course of this current was changed by ADP, the entire effect being reversible on withdrawing the agonist (Figure 1, insert). The mean from such experiments on 16 cells for the maximum inhibition (produced by an application of 10 μM ADP) was 64.0 ± 1.7%. The maximum inhibition in control cells was 13.9 ± 3.7% ($n=10$), indicating the presence of a low level of endogenous $P2Y_1$-like receptors in SCG neurones, as found previously (Filippov *et al*., 1998) to be specifically activated by ADP and ATP.

The concentration-dependence of ADP (Figure 1) fitted the Hill equation with an EC_{50} value of 8.6 ± 1.3 nM ($n_H=0.95\pm0.12$). This curve was right-shifted by the presence of the $P2Y_1$ antagonist adenosine-3′, 5′-*bis*-phosphate (Figure 1), with $K_i=0.7$ μM, in good agreement with the value (K_i 1 μM) found for this agent by Boyer *et al*. (1996) on the human $P2Y_1$ receptor heterologously expressed in the astrocytoma cell line.

The substitution of a methylthio (MeS) group at N2 of the adenine ring has been well established to increase activity strongly in $P2Y_1$ agonists in other transductions, as well as in the receptor binding (Simon *et al*., 1995; Schachter *et al*., 1996). We tested 2-MeSADP in the Ca^{2+}-channel response and found that it behaved identically to ADP, but with potency increased about 15 fold (Figure 2). Indeed, the EC_{50} value of 0.57 ± 0.05 nM is the highest potency found in any $P2Y_1$ transduction for this commonly tested agonist.

To examine whether the triphosphates are antagonists (see Introduction) in this system, 2-MeSATP was tested in the same conditions. It gave a Ca^{2+} current inhibition identical in form to that shown for ADP in Figure 1 (insert), and was a very potent agonist (Figure 2), with an EC_{50} value of 2.5 ± 0.3 nM ($n_H=1.00\pm0.09$). 2-MeSATP was, however, consistently a partial agonist, the difference in the maximum response values for this and 2-MeSADP, as illustrated in Figure 2, being statistically significant. For ATP, full concentration-response plots could not be constructed because at the higher concentrations ATP (unlike the other agonists used) opened P2X channels present (Cloues *et al*., 1993) on the SCGs.

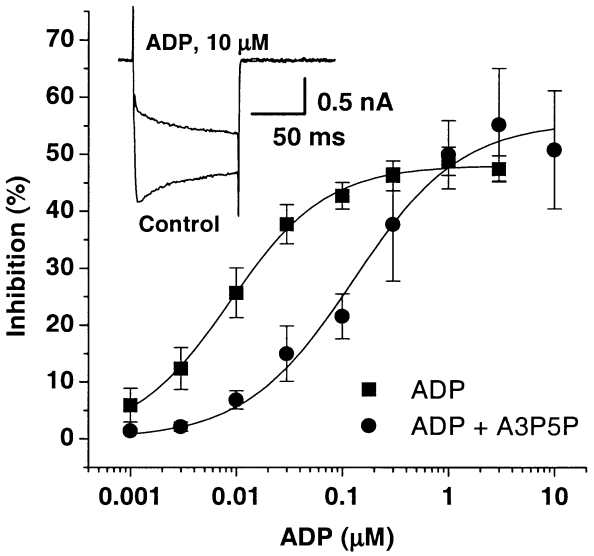

Figure 1 Concentration-response curves for the inhibition of the N-type Ca^{2+} current by ADP. Each curve is fitted by the Hill equation to the means (shown, with s.e. bars) of the normalized data points from five $P2Y_1$-expressing SCG neurones. ADP was applied in the absence or the presence of the $P2Y_1$ antagonist, A3P5P (10 μM). (The difference between the maximum responses here is not significant). *Insert*: A representative recording of the Ca^{2+} current (leak-subtracted) in the absence and presence of ADP. Current recorded on a 100 ms test pulse from −90 mV to 0 mV and measured 10 ms after beginning of the test pulse (see Filippov *et al*., 1998 for details).

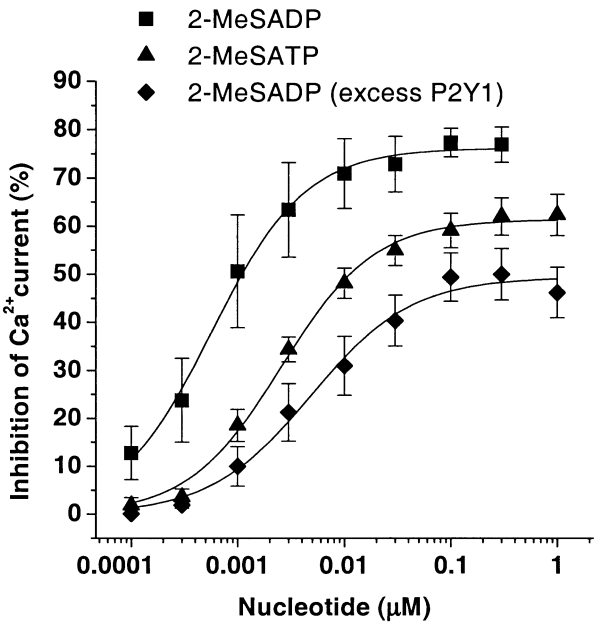

Figure 2 Concentration-response curves (constructed as for Figure 1) for 2-MeSADP (squares) or 2-MeSATP (triangles); also shown (lowest curve) response to 2-MeSADP when excess $P2Y_1$ cDNA was injected and the final incubation period was doubled (for details, see text).

However, ATP was again clearly agonistic at P2Y1, and at 1 μM (below its threshold for P2X activation) gave a range of 65–77% of the response to 1 μM ADP when both were tested on the same cells.

Since the diphosphates are strong agonists, if they were present in the triphosphates this could mimic agonist activity of the latter, although this effect would be small at the known low levels of that impurity in the sources used here and would become very small if the triphosphate were in fact an antagonist (Fagura *et al*., 1998). To eliminate this possibility the triphosphates were treated with creatine kinase plus creatine phosphate (Hechler *et al*., 1998) prior to use, a treatment shown to convert quantitatively any diphosphate present to the triphosphate, and all the results on the latter were obtained in that state. However, a possibility still remained that, in placing the 2-MeSATP in solution and performing all of the sequential recordings on a single cell for an individual concentration-response curve, some subsequent conversion to the diphosphate could nevertheless occur. Therefore, this was tested by maintaining the creatine kinase mixture throughout in the agonist reservoir; applications of 10 nM 2-MeSATP were made such that contact of this enzyme-containing agonist solution with each cell was limited to 20 s. This nevertheless gave a current inhibition of 51.6%, an agonist activity within the range found at that concentration (Figure 2) without that added precaution. The presence of, or conversion to, diphosphate was therefore discounted as an explanation of these results.

Since a higher receptor density may strongly increase the potency measured for a G-protein-coupled receptor (Kenakin, 1997), an issue here, we tested the effect of a higher receptor density in the cell than is produced in the standard conditions used in these experiments. The quantity of $P2Y_1$ cDNA injected into the nucleus was increased about as far as was feasible, by 3.3 fold, and the post-injection period before applying agonist was doubled, a change which normally leads to a considerable further accumulation of receptors at the cell membrane. These changes led, not to an increased response of each cell, but to a statistically significant decrease of about one-third in the maximum response, and much greater decreases at lower agonist concentrations (Figure 2). The EC_{50} value was increased about 9 fold, to 4.8 ± 0.7 nM ($n_H = 0.92 \pm 0.10$). Although it was not feasible to determine the densities of receptors present at the cell membrane in the neurones recorded, these tests suggested that in our standard conditions the $P2Y_1$ receptor density at the membrane is already at or near the maximum attainable by ectopic expression in these cells, and that when greater numbers are produced receptor internalization and down-regulation are stimulated so that the net density is lowered. That pathway is well established for the β2-adrenergic receptor, where a similar decreased response has been found with over-expression (Zhong *et al*., 1996).

Discussion Using identified native neurones as host cells, it was possible to reveal a transduction event at the cell membrane – the closing of the N-type Ca^{2+} channel – that is powerfully evoked by $P2Y_1$ activation. We propose that this is a physiologically relevant action of $P2Y_1$ in neurones, since: (a) $P2Y_1$ is the only P2Y receptor subtype that has been found to be abundant in neurones in many locations (see Introduction); (b) the N-type Ca^{2+} channel is characteristic of neurones, and is involved presynaptically in neurotransmitter release and postsynaptically in neuronal excitation (as discussed by Filippov *et al*., 1998); (c) the receptor density attained here seems appropriate for such functions, since the maximum inhibition of the Ca^{2+} current obtainable (at saturating 2MeSADP) is, while high, still incomplete and only slightly greater than the maximum obtainable on the same cells with noradrenaline *via* their native α_2-adrenoceptors and (d) these rat SCG neurones exhibit in a much lower amount a *native* $P2Y_1$-like receptor response where ADP and ATP act likewise to close this channel (Filippov *et al*., 1998).

This action is additional to the known pathway of $P2Y_1$-evoked release of Ca^{2+} from internal stores, which occurs in a very wide range of cell types. The agonist potency and selectivity for the latter activity, as measured on a $P2Y_1$-transfected non-neuronal cell line (Palmer *et al*., 1998), are similar to those found here for the neuronal Ca^{2+} channel transduction.

We have also found evidence that the adenosine triphosphates can be strong agonists, rather than antagonists, at $P2Y_1$, contrary to much current discussion. This is not due to the different transduction studied nor to the specific neuronal environment, since the same finding was made (Palmer *et al*., 1998) for the Ca^{2+} mobilization by $P2Y_1$ in transfected non-neuronal cells. Although we have studied the rat $P2Y_1$, our finding does not arise from species differences in the protein sequence, since the two conflicting results on the triphosphates (see Introduction) were both obtained on the human $P2Y_1$ form. Palmer *et al*. (1998) proposed that this discrepancy might arise because $P2Y_1$ may be expressed at a much higher level in the 1321N1 host cells which they used than in the Jurkat cells used by the other groups cited above. Evidence for this came from the predicted effect of decreasing the functional receptor density by desensitization and also from the 20 fold greater potency of 2-MeSADP and of ADP in the former compared to the latter case. As analysed by Kenakin (1997), a change from a low to a very high receptor reserve could give a great increase in agonist potency, and even convert a ligand of lower intrinsic activity from apparent antagonist or inverse agonist to true agonist activity. In our cells those two ligands give even stronger agonist potency than reported for the 1321N1 cells as host, or for any other host. This, together with the high efficiency of expression to be expected from the direct microinjection into the individual cell to be studied, leads us to suggest that a relatively high receptor density is indeed generated at the membrane of our cells. This could explain the previous differing observations discussed above. This effect has been demonstrated in practice, for some receptor coupling situations. Thus, Zhong *et al*. (1996) and Hermans *et al*. (1999) applied an inducible promoter to obtain controlled increases in the density of, respectively, β-adrenoceptors or a metabotropic glutamate receptor, to show increases in agonist potencies, up to 50 fold with β1 receptors. Also, as receptor theory predicts, a partial agonist was dramatically increased in efficacy, while antagonist activities were unchanged (Hermans *et al*., 1999). Thus, for $P2Y_1$, ATP cannot be a true antagonist in any conditions.

A second factor (which might enhance the effect of the first) is that $P2Y_1$ desensitization (Palmer *et al*., 1998) might occur from the constant release of ATP from, e.g., the Jurkat cells used at high cell density. This is unlikely to arise in the two continuously-superfused low cell density systems on cover-slips of Palmer *et al*. (1998) and of ourselves. That factor would affect ATP, as a poorer agonist, much more than ADP.

Yet a third possibility is that a high receptor density present in our cells favours the association of $P2Y_1$ proteins, e.g. to form homodimers, a structure recently found in a range of G-protein-coupled receptors, where it can sometimes change ligand affinities greatly (reviewed by

Hebert & Bouvier, 1998). As yet this has not been investigated in the P2Y series. If this occurs it could amplify the aforementioned receptor reserve effect of receptor density differences to produce a greater sensitivity to the agonists including ATP.

Higher local receptor densities occur on neurones at synapses. Overall, we conclude that ATP as well as ADP could be a potent agonist at $P2Y_1$ receptors at synapses and thereby inhibit N-type Ca^{2+} currents, whereas $P2Y_1$ receptors elsewhere at low densities are attuned to recognize only its breakdown product, ADP.

We thank Dr T.E. Webb for the rat $P2Y_1$ cDNA and Dr J. Simon (Cambridge) for preparing the $P2Y_1$-vector used. This work was supported by the Wellcome Trust.

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(Received December 2, 1999
Accepted December 21, 1999)