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Enhancement of the response to purinergic agonists in $P2Y_1$ transfected 1321N1 cells by antagonists suramin and PPADS

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1 We have previously shown that both suramin and pyridoxal-phosphate-6-azophenyl-2′, 4′ disulphonic acid (PPADS) act as antagonists at transfected $P2Y_1$ receptors. Here we show that under certain experimental conditions these two P2 antagonists can enhance the response to agonists acting at these receptors.

2 The expression of either $P2Y_1$ or $P2Y_2$ receptors in 1321N1 human astrocytoma cells results, on a change of medium, in an elevation of basal (no added agonist) accumulation of [^{3}H]-inositol(poly)phosphates([^{3}H]-$InsP_x$) compared to cells not expressing these receptors. This elevation is much greater in $P2Y_1$ transfectants than in $P2Y_2$ transfectants.

3 Both PPADS and suramin reduced this basal level of [^{3}H]-$InsP_x$ accumulation in the $P2Y_1$ expressing cells.

4 When a protocol was used which required changing the culture medium, antagonists were added at a concentration which reduced the basal accumulation by about 50%, there was a significant stimulation in response to increasing concentrations of 2-methylthioadenosine 5′-triphosphate (2MeSATP), in the absence of antagonists there was no significant effect of the agonist.

5 However, when 2MeSATP was added in the absence of a change of medium and with no antagonist present, there was a several fold increase in [^{3}H]-$InsP_x$ accumulation. These results show that a release of endogenous agonist activity (possibly ATP/ADP) from the $P2Y_1$ expressing cells can create conditions in which a response to an agonist such as 2MeSATP can only be seen in the presence of a competitive antagonist.

Keywords: P2 receptors; P2Y receptors; purinoceptors; $P2Y_1$receptors; pyridoxal-phosphate-6-azophenyl-2′,4′disulphonic acid (PPADS); suramin; 2-methylthioATP (2MeSATP)

Introduction

The G protein-coupled cell surface receptors for adenosine 5′-triphosphate (ATP)/adenosine 5′-diphosphate (ADP) and uridine 5′-triphosphate (UTP)/UDP, collectively referred to as the P2Y nucleotide receptors (Fredholm *et al*., 1994), play a widespread role in regulation of cellular function (Boarder *et al*., 1995). These nucleotides act as mediators of communication between cells, being released from one cell and controlling others. Our understanding of these events has been enhanced by cloning of members of this receptor family, now extending from $P2Y_1$ to $P2Y_7$ (e.g. Webb *et al*., 1993; Lustig *et al*., 1993; Filtz *et al*., 1994; Parr *et al*., 1994; Tokuyama *et al*., 1995; Henderson *et al*., 1995; Nguyen *et al*., 1995; Communi *et al*., 1995; 1996; Akbar *et al*., 1996). Recent studies in which the phospholipase C (PLC) response was measured in transfected human 1321N1 astrocytoma cells, have described the characterization of $P2Y_1$ (formerly P_{2Y}), $P2Y_2$ (P_{2U}) and $P2Y_4$ with respect to antagonism by the previously described P2 antagonists, pyridoxal-phosphate-6-azophenyl-2′,4′disulphonic acid (PPADS) and suramin (e.g. Hoyle *et al*., 1990; Lambrecht *et al*., 1992; Brown *et al*., 1995). The results showed that these receptors exhibit differential sensitivity to these antagonists (Charlton *et al*., 1996a, b). Studies preliminary to these provided two observations. When stimulation involved a complete change of medium in order to add the agonist there was little or no response of the $P2Y_1$ transfectants to the agonist 2-methylthioATP (2MeSATP). Secondly, under these conditions the 'basal' level of phospholipase C (PLC) activity (i.e. no added agonist) could be reduced by the presence of the P2 antagonist suramin. While the latter result might be taken to suggest the presence of constitutive activity in the cloned receptors, the studies described in the present study support the view that these results are caused by the release of an endogenous agonist for the $P2Y_1$ receptor (see Lazarowski *et al*., 1995), and define conditions in which the presence of an antagonist can enhance the responses to an agonist.

Methods

Lines of 1321N1 cells stably transfected with turkey $P2Y_1$ (t-$P2Y_1$) and human $P2Y_2$ (h-$P2Y_2$) cells, donated by G.A. Weisman, J.T. Turner and L. Erb (University of Missouri-Columbia), have been described previously (Parr *et al*., 1994; Filtz *et al*., 1994; Charlton *et al*. 1996a) and were used essentially as indicated in Charlton *et al*. (1996a). Cells were used in 24 well multiwells just before they reached confluence. They were labelled by preincubation for 24 h at 37°C with 0.5 ml of 1 μCi ml^{-1}[2-^{3}H]-inositol in medium M199. Following this the medium was aspirated, the cells washed in 1 ml balanced salt solution (BSS in mM: NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, HEPES 30, NaH_2PO_4 1, $MgSO_4$ 0.8, $CaCl_2$ 1.8 and glucose 5.5, pH 7.4) and then replaced with 0.45 ml BSS containing 10 mM LiCl for 10 min. At this time 50 μl of agonist at 10 times final concentration was added, followed by an incubation period of 15 min at 37°C. This incubation was terminated by aspiration and immediate addition of 0.5 ml of cold 0.5 M trichloroacetic acid. Cell extracts were then left on ice for 1 h before extraction of the total [^{3}H]-inositol (poly)phosphates ([^{3}H]-$InsP_x$) on small Dowex-1 (Cl^-) columns. Antagonists were added, where appropriate, with LiCl.

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Hexokinase was used in some cases when ADP was the agonist in order to remove any ATP contaminating the purchased ADP or formed from ADP during the incubation with the cells (Nicholas *et al*., 1996). Stock solutions of ADP (5 mM) were preincubated with 50 units ml^{-1} hexokinase (Boehringer-Mannheim) and 110 mM glucose for 1 h, and hexokinase (1 unit ml^{-1}) and glucose (22 mM) were present with the cells for 10 min before and during the incubation with the agonist.

Results

By use of the protocol described above the expression of the t-$P2Y_1$ receptor in 1321N1 cells was found to raise the accumulation of [^{3}H]-$InsP_x$ in cells to which no agonist was added. This is shown in Figure 1a when this 'basal' level of accumulation was expressed as fold over that seen with cells transfected with the vector without the $P2Y_1$ insert. The expression of the $P2Y_2$ receptor was expressed in these cells there was also an elevation in basal accumulation of [^{3}H]-$InsP_x$, but in this case this was a relatively modest increase of approximately 3 fold. This was unlikely to be due to a gross difference in the number of functional receptors expressed by the 2 systems, since under different conditions of stimulation there was a similar maximal response to agonists in the two transfected cell lines (Charlton *et al*., 1996a). This explanation is also rendered unlikely by the results shown in Figure 1b, in which the effect of stimulation of the $P2Y_1$ and $P2Y_2$ cells with maximally effective concentrations of the relevant agonists (2MeSATP and UTP respectively) shows that the $P2Y_2$ transfectants gave a larger response (expressed as fold over basal) than the $P2Y_1$ transfectants. The low fold/basal stimulation for $P2Y_1$ seen in Figure 1b is a consequence of the high basal value seen in Figure 1a. This is also suggested by the data from individual experiments: e.g. for $P2Y_1$ transfectants, control 3189 d.p.m. and 3 μM 2MeSATP 4709 d.p.m.; for $P2Y_2$ transfectants, control 1600 d.p.m. and 100 μM UTP 6050 d.p.m.; in each case data are from a single experiment and are the mean of triplicate results.

Figures 2 and 3 show that PPADS and suramin can both inhibit the basal level of [^{3}H]-$InsP_x$ accumulation in the $P2Y_1$ transfected cells. In each case a concentration of antagonist was chosen which in preliminary experiments had been shown to generate approximately 50% inhibition of the basal level of [^{3}H]-$InsP_x$ accumulation. The apparent reduction in PLC activity could be the result of constitutive activity in the transfected $P2Y_1$ receptor, with PPADS and suramin acting as antagonists with negative efficacy. However, our results show that the apparent constitutive activity is dependent on the conditions used for stimulation. If, instead of changing the medium at the end of the labelling period, as described above, the additions were made to the labelling medium directly without disturbing the cells, then the basal value was considerably reduced (e.g. from 2940 ± 295 d.p.m. of [^{3}H]-$InsP_x$ when the medium was changed to 384 ± 46 d.p.m. ($n=3$) when there was no change of medium). The fold stimulation in response to 3 μM 2MeSATP was increased from 1.51 ± 0.02 fold over basal when the medium was changed to 4.18 ± 0.17 fold over basal in the absence of a medium change ($n=3$). In the absence of a change in medium with no added agonist suramin and PPADS induced a small effect with the $P2Y_1$ transfectants,

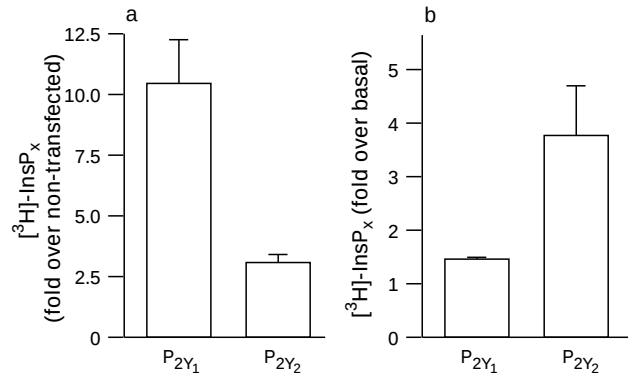

Figure 1 (a) Basal accumulation of [^{3}H]-$InsP_x$ in $P2Y_1$ and $P2Y_2$ transfected 1321N1 cells expressed as fold over the basal in cells not expressing the P_2 receptors. (b) Accumulation of [^{3}H]-$InsP_x$ in $P2Y_1$ and $P2Y_2$ transfected cells in response to 3 μM 2MeSATP ($P2Y_1$) or 100 μM UTP ($P2Y_2$), expressed as fold over basal (no added agonist). In each case results are mean $\pm$ s.e.mean, $n=3$ experiments each in triplicate.

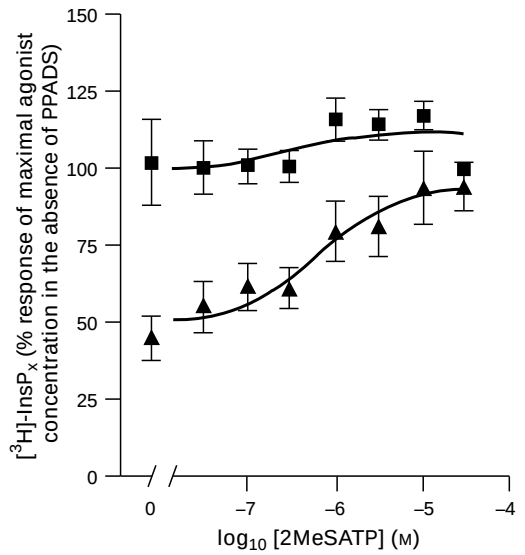

Figure 2 Effect of 30 μM PPADS on the concentration-response curve to 2MeSATP of 1321N1 cells expressing $P2Y_1$ receptors. The effect of increasing concentrations of 2MeSATP in the absence of PPADS (■) had no significant effect on [^{3}H]-$InsP_x$ accumulation, while in the presence of PPADS (▲) there was a significant effect of 2MeSATP ($P<0.0003$, by analysis of variance). Data are mean, $n=5$ separate experiments each in triplicate; vertical lines show s.e.mean.

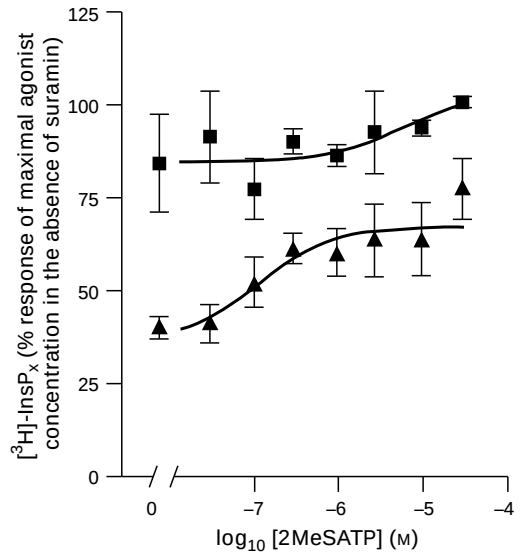

Figure 3 Effect of 10 μM suramin on the concentration-response curve to 2MeSATP of 1321N1 cells expressing $P2Y_1$ receptors. The effect of increasing concentrations of 2MeSATP in the absence of suramin (■) had no significant effect on [^{3}H]-$InsP_x$ accumulation, while in the presence of suramin (▲) there was a significant effect of 2MeSATP ($P<0.001$, by analysis of variance). Data are mean, $n=3$ separate experiments each in triplicate; vertical lines show s.e.mean.

but had no effect with the $P2Y_2$ transfectants. These results support the view that the high basal values were not caused by constitutive activity, but by the presence of agonist released from the cell on change of medium, and that the levels of endogenous extracellular agonist are considerably reduced by not changing the medium after labelling (Lazarowski *et al*., 1995).

The difference between $P2Y_1$ and $P2Y_2$ transfectants with respect to response on changing the medium was probably caused by differential responsiveness to the endogenous agonists released from the cells. The most likely candidate in terms of this differential response is ADP (see Discussion). During this series of experiments the EC_{50} for ATP at the $P2Y_1$ transfectants was determined to be 2.6 μM and for ADP was 0.42 μM. At the $P2Y_2$ transfectants the EC_{50} for ATP was 0.5 μM and the apparent EC_{50} for ADP was 4.2 μM. In the presence of hexokinase, which removes contaminating ATP (Nicholas *et al*., 1996), the EC_{50} for ADP with the $P2Y_2$ transfectants became greater than 40 μM, indicating that ADP is essentially inactive at the $P2Y_2$ receptor, but a potent agonist at the $P2Y_1$ receptor. Conversely, ATP was more potent at the $P2Y_2$ receptor than at the $P2Y_1$ receptor.

When concentration-response curves to the $P2Y_1$ agonist 2MeSATP were constructed in the presence and absence of a level of PPADS which reduced the basal levels of ^{3}[H]-InsP_x by about 50%, results were obtained which are presented in Figure 2. There was little or no response to the agonist in the absence of PPADS, consistent with the very limited fold over basal stimulation described above and in Figure 1b. The presence of 30 μM PPADS created conditions in which a concentration-response curve could be constructed for 2MeSATP between the range 10^{-7} and 10^{-5} M. Similar experiments were undertaken with suramin. Figure 3 shows that in the absence of antagonist there was essentially no concentration-response curve, while in the presence of 10 μM suramin there was a loss of basal values to approximately 50%, and a response curve to 2MeSATP between 10^{-7} and 10^{-5} M. Higher concentrations of suramin shifted the curve to 2MeSATP to the right and resulted in a smaller response to highest concentration of 2MeSATP used (data not shown).

Discussion

In this study we have shown that the presence of an antagonist can, under certain circumstances, increase the response to an agonist. The requirements for this are: firstly, activity in the absence of added agonist; secondly, a competitive mode of action of the antagonist, such that the effect of the antagonist can be surmounted by suitable concentrations of agonist. There are at lease two sets of conditions which can create this situation. One is a receptor with intrinsic activity and an antagonist with negative efficacy. The alternative is release of an endogenous agonist by the cells or tissue under study. One way to distinguish between these possibilities is the investigation of the presence of endogenous agonist. In the case of 1321N1 cells, the host cells in the present study, it has previously been shown that they release nucleotide agonists in response to changes of the medium (Lazarowski *et al*., 1995). In this study we have shown that with $P2Y_1$ transfected 1321N1 cells the basal levels of ^{3}[H]-InsP_x were substantially reduced in the absence of a change of medium. Furthermore, the basal levels in the $P2Y_1$ transfected 1321N1 cells were affected by the presence of PPADS (Figure 2) and by the presence of suramin (Figure 3). This is interpreted, in the light of the above, as antagonism of the action of endogenous agonist.

Some indications are available concerning the likely nature of the endogenous agonist activity. Lazarowski *et al*. (1995) have shown that both ATP and ADP are present in the medium of 1321N1 cells, although their studies only investigated adenine nucleotides, and would therefore not measure UTP or UDP. However, the endogenous agonist activity has a relatively small effect on the $P2Y_2$ expressing cells. Since UTP and UDP are more effective agonists at the $P2Y_2$ receptor than at the $P2Y_1$ receptor, the latter observation suggests that the endogenous agonist activity is not predominantly UTP/UDP. The simplest explanation is that the endogenous activity is mainly ATP/ADP. However, since ATP is effective at both $P2Y_2$ and $P2Y_1$ receptors, it seems likely that this is not the principal agonist. ADP, which is selective for $P2Y_1$ receptors, over $P2Y_2$ receptors (Boarder *et al*., 1995; Nicholas *et al*., 1996; this paper), remains a likely candidate, although other agonists showing preferential activation of the $P2Y_1$ receptors cannot be excluded.

The observation of little or no response to agonist in $P2Y_1$ transfected 1321N1 cells in the absence of antagonist implies that the level of endogenous agonist is sufficient to generate a maximum response. The concentration of PPADS and suramin used were sufficient to reduce this activity effectively, and yet low enough to be competed out when further exogenous agonist was added. In this way a response to the agonist was seen only in the presence of the antagonist.

These results reveal a novel and somewhat unexpected consequence of the presence of a competitive antagonist. It is possible that this pattern of events might be reflected by cells *in situ*. Where the tone on a system is naturally high, then the further presence of agonist will have no effect (e.g. increase in rate of release of neurotransmitter). However, if the effective tone had been reduced previously by administration of a competitive antagonist, then fluctuations in the level of the agonist (e.g. fluctuations in the release of neurotransmitter) may determine the size of response generated by the cells.

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