# ADP stimulation of inositol phosphates in hepatocytes: role of conversion to ATP and stimulation of $P2Y_2$ receptors

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**1**  Accumulation of inositol (poly)phosphates ($InsP_x$) has been studied in rat hepatocytes labelled with [³H]inositol. Stimulation with ADP resulted in a significant increase in total [³H]$InsP_x$, whereas 2-MeSADP had only a small effect and ADP$\beta$S was ineffective. UTP and ITP also stimulated substantial increases in [³H]$InsP_x$.

**2**  The dose–response curve to ADP was largely unaltered by the presence of the $P2Y_1$ antagonist, adenosine-3′-phosphate-5′-phosphate (A3P5P). Similarly, inclusion of MRS 2179, a more selective $P2Y_1$ antagonist, had no effect on the dose–response curve to ADP.

**3**  The inclusion of hexokinase in the assay reduced, but did not abolish, the response to ADP.

**4**  HPLC analysis revealed that ADP in the medium was rapidly converted to AMP and ATP. The inclusion of hexokinase removed ATP, but exacerbated the decline in ADP concentration, leading to increased levels of AMP. 2-MeSADP was stable in the medium and ATP was largely unaffected.

**5**  The addition of the adenylate kinase inhibitor, diadenosine pentaphosphate ($Ap_5A$) significantly reduced the ADP response. HPLC analysis conducted in parallel demonstrated that this treatment inhibited conversion of ADP to ATP and AMP.

**6**  Inclusion of the P1 antagonist CGS 15943 had no effect on the dose–response curve to ADP.

**7**  These observations indicate that hepatocytes respond to ADP with an increase in inositol (poly)phosphates following conversion to ATP. $P2Y_1$ activation in hepatocytes does not appear to be coupled to inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) production.

## Introduction

P2Y receptors comprise a family of G-protein-coupled receptors responding to the native nucleotides ADP, ATP, UDP and UTP (Burnstock, 1996). In common with many other cell types rat hepatocytes co-express multiple P2Y subtypes, although these have not been conclusively identified (Dixon *et al.*, 1990; 2000; Keppens, 1993). Reverse transcriptase-polymerase chain reaction (RT–PCR) has demonstrated the presence in primary rat hepatocytes of mRNA transcripts encoding all four of the cloned rat P2Y subtypes investigated ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$; Dixon *et al.*, 2000). However, $P2Y_6$ receptors appear not to be expressed, as UDP (the most potent agonist at this receptor subtype; Nicholas *et al.*, 1996) had no effect on intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in rat hepatocytes (Dixon *et al.*, 2000). Similarities in the responses to ATP and UTP have indicated that these nucleotides act at a common receptor (Dixon *et al.*, 2000; Keppens *et al.*, 1992). On the basis of sensitivity of the responses to suramin, we have argued for

expression of $P2Y_2$ and not $P2Y_4$ receptors (Dixon *et al.*, 2000); rat $P2Y_2$ receptors are inhibited by suramin (Chen *et al.*, 1996) whereas rat $P2Y_4$ receptors are unaffected (Bogdanov *et al.*, 1998). Of the more recently identified members of the P2Y family ($P2Y_{11-13}$), the rat homologue of $P2Y_{12}$ has been cloned but its presence not determined in hepatocytes. Rat $P2Y_{11}$ and $P2Y_{13}$ receptor clones have not yet been reported.

In single perfused rat hepatocytes, the transients in $[Ca^{2+}]_i$ generated in response to ADP, 2-methylthioADP (2-MeSADP) and 2-methylthioATP (2-MeSATP) are indistinguishable, indicating a common receptor (Dixon, 2000; Dixon *et al.*, 1995). The recently cloned canine $P2Y_{11}$ receptor is activated by these agonists (Qi *et al.*, 2001; Torres *et al.*, 2002; Zambon *et al.*, 2001). Studies of $P2Y_{11}$ receptors show a species difference; the human homologue is activated most effectively by triphosphates (Communi *et al.*, 1997) whereas the canine receptor responds to nucleotide diphosphates (Qi *et al.*, 2001; Torres *et al.*, 2002; Zambon *et al.*, 2001). As the rat $P2Y_{11}$ receptor has yet to be cloned its agonist profile is not known. It must, however, be considered as a possible mediator of the effects of 2-MeSADP, 2-MeSATP and ADP.

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Functional data, however, suggest that these responses are mediated by P2Y$_1$ receptors (Dixon *et al*., 1995; 2000; Dixon, 2000). Thus the [Ca$^{2+}$]$_i$ responses of single cells to both 2-MeSADP and 2-MeSATP were blocked by the P2Y$_1$ antagonists, PPADS (Dixon *et al*., 2000) and adenosine-3'-phosphate-5'-phosphate (A3P5P; Dixon, 2000). Surprisingly, the [Ca$^{2+}$]$_i$ response to 2-MeSATP in rat hepatocytes occurred in the absence of detectable increases in inositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$) (Keppens & De Wulf, 1991). This was in contrast to ADP which produced a substantial Ins(1,4,5)P$_3$ response (Keppens & De Wulf, 1995). These observations are difficult to reconcile: while ADP, 2-MeSADP and 2-MeSATP are believed to act on a common P2Y$_1$ receptor, only ADP stimulates PLC. However, in the earlier Ins(1,4,5)P$_3$ experiments, consideration was not given to the possible effects of agonist interconversion in the recorded responses.

Here we have investigated the accumulation of total [$^3$H]-inositol (poly)phosphates ([$^3$H]-InsP$_x$) in response to the P2Y$_1$ agonists, ADP, 2-MeSADP and ADP$\beta$S, and have studied the effects of the P2Y$_1$ antagonist, A3P5P (Boyer *et al*., 1996) and its highly selective derivative, MRS 2179 (Boyer *et al*., 1998). The effects of ATP, UTP and ITP are also reported. In parallel experiments HPLC analysis revealed that the response to ADP was likely to be secondary to its conversion to AMP and ATP through the action of an ecto-adenylate kinase. Consistent with this, inclusion of the adenylate kinase inhibitor, Ap$_5$A (Lienhard & Secemski, 1973) greatly reduced the effect of ADP.

# Methods

## Cell preparation and culture

Hepatocytes were isolated from fed, male Wistar-strain rats (150 – 250 g) by collagenase perfusion as described previously (Dixon *et al*., 1995). Briefly, the rat was killed by cervical dislocation in accordance with institutional guidelines, and the hepatic portal vein cannulated. An initial Ca$^{2+}$-free perfusion was followed by perfusion with collagenase (0.04% w v$^{-1}$) and Ca$^{2+}$ (3.8 mM) for 15 min. The perfusion rate was 30 ml min$^{-1}$ throughout. The cells were harvested and cultured for 24 h at a density of $1 \times 10^5$ cells well$^{-1}$ in 24-well plates in William's medium E with 10% FCS, 50 iu ml$^{-1}$ penicillin, 50 $\mu$g ml$^{-1}$ streptomycin and 50 $\mu$g ml$^{-1}$ gentamicin.

## Total [$^3$H]-inositol (poly)phosphates

Cells were labelled for 48 h at 37°C in 5% CO$_2$ with *myo*-[2-$^3$H]inositol (0.037 Mbq ml$^{-1}$, 0.5 ml well$^{-1}$) in Medium M199 with the same additions of antibiotics. Twenty min stimulations, in the presence of 10 mM LiCl, were made without change of medium. In experiments with the P2Y$_1$ antagonists, A3P5P and MRS 2179 (100 $\mu$M), the P1 antagonist, CGS 15943 (1 $\mu$M), and the adenylate kinase inhibitor, Ap$_5$A (300 $\mu$M), cells were pre-incubated for 10 min prior to agonist addition. Hexokinase was included at 3 units well$^{-1}$ where indicated. Total [$^3$H]-InsP$_x$ were extracted on small Dowex-1 (Cl$^-$) columns.

## HPLC analysis

In parallel experiments medium was collected from above cells in 24-well plates following the 20 min stimulation period under identical experimental conditions as the [$^3$H]-InsP$_x$ assay. HPLC analysis of the nucleotide content was conducted to determine the extent of agonist interconversion. Nucleotides were identified by co-chromatography.

## Statistical analysis

Data presented in figures are representative of three experiments (mean ± s.e.mean) from different hepatocyte preparations and expressed as d.p.m. after subtraction of controls. Data pooled across experiments are presented in the text; statistical analysis was by two-way ANOVA with Bonferroni's post-test. Since the dose – response curves did not saturate, true EC$_{50}$ values could not be reported. Instead, we report apparent EC$_{50}$ values, calculated as the concentration of agonist which generates 50% of the response to the maximal concentration used.

## Materials

Cell culture medium was from GIBCO BRL (Paisley, U.K.). *Myo*-[2-$^3$H]inositol was purchased from Amersham Pharmacia Biotech (Little Chalfont, Bucks, U.K.). Collagenase was from Boehringer (Lewes, U.K.). Hexokinase, nucleotides, A3P5P, MRS 2179, Ap$_5$A, CGS 15943 and all other chemicals were from Sigma-Aldrich (Poole, U.K.). 10 mM stock solutions of ADP, ADP$\beta$S and Ap$_5$A were incubated for 1 h with 80 units ml$^{-1}$ hexokinase and 20 mM glucose (ADP) and 20 units ml$^{-1}$ grade VII apyrase (ADP$\beta$S and Ap$_5$A) and HPLC analysis used to confirm purity. The apyrase-treated Ap$_5$A stock was then heated to 100°C for 10 min to remove apyrase activity. Samples of ADP and ATP (300 $\mu$M) were incubated for 20 min at 37°C with 1 mM heat-treated Ap$_5$A, and HPLC analysis used to ensure that the apyrase activity had been destroyed.

# Results

Stimulation of [$^3$H]-inositol-labelled hepatocytes with ADP or ATP led to a concentration-dependent accumulation of [$^3$H]-InsP$_x$. Figure 1 shows that at the same concentration the response to ADP was significantly smaller than the response to ATP (data pooled across three experiments, $P < 0.05$ by two-way ANOVA). At the maximal concentration used (300 $\mu$M) the ADP response was 76.7 ± 7.3% ($n = 3$ separate experiments) of the ATP response ($P < 0.05$, Bonferroni's post-test). Whilst potencies cannot be determined for responses that do not form a plateau, the apparent EC$_{50}$ values, assuming that 300 $\mu$M agonist gave a maximal response, were ATP, 21 $\mu$M (log EC$_{50}$ = $-4.7 \pm 0.1$) and ADP, 63 $\mu$M (log EC$_{50}$ = $-4.3 \pm 0.2$). These apparent EC$_{50}$ values for ATP and ADP were not significantly different. In contrast to the effect of ADP, 2-MeSADP yielded only a small [$^3$H]-InsP$_x$ response as shown in Figure 1 (16.7 ± 3.2% of the ATP response at 300 $\mu$M; $n = 3$). ADP$\beta$S treated with apyrase to remove contaminating ADP and ATP, was ineffective (6.5 ± 2.8% of the ATP response at 300 $\mu$M; $n = 3$; Figure 1).
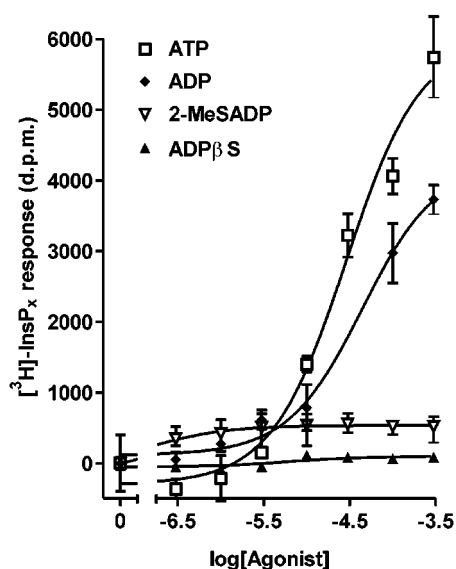
**Figure 1** Effect of ADP, ATP, ADP$\beta$S and 2-MeSADP on inositol (poly)phosphate accumulation in rat hepatocytes. Total [³H]InsP$_x$ accumulation was measured in [³H]inositol-labelled cells in response to incubation for 20 min with increasing concentrations of agonist. Results, shown as d.p.m. after subtraction of unstimulated controls, are from a single representative experiment, with stimulations performed in triplicate. Data pooled from three separate experiments are reported in the text.
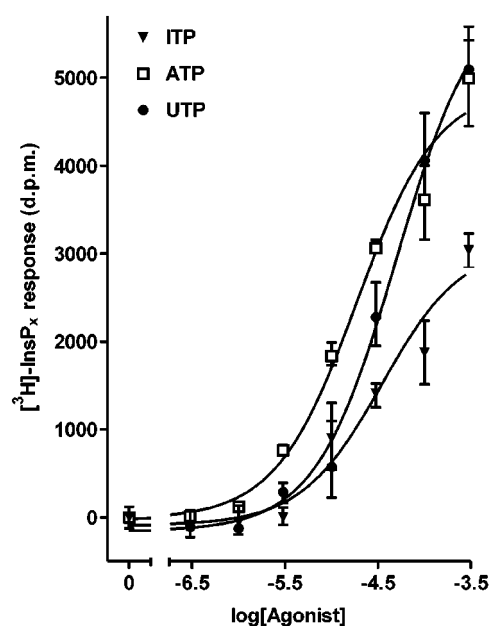


**Figure 2** Effect of ATP, UTP and ITP on inositol (poly)phosphate accumulation in rat hepatocytes. Total [³H]InsP$_x$ accumulation was measured in [³H]inositol-labelled cells in response to incubation for 20 min with increasing concentrations of agonist. Results, shown as d.p.m. after subtraction of unstimulated controls, are from a single representative experiment, with stimulations performed in triplicate. Data pooled from three separate experiments are reported in the text.

As shown in Figure 2, substantial increases in [³H]-InsP$_x$ were measured in response to UTP and ITP. The response to the maximal UTP concentration was indistinguishable from that to ATP, pooled across three separate experiments. However, the response to ITP was significantly different from ATP ($P<0.001$ by two-way ANOVA of pooled data); 300 $\mu$M ITP yielded $83.7\pm8.5\%$ ($n=3$) of the response to 300 $\mu$M ATP. The apparent EC$_{50}$ values for ATP and ITP were not significantly different; ATP, 15 $\mu$M (log EC$_{50}=-4.9\pm0.1$) and ITP, 60 $\mu$M (log EC$_{50}=-4.3\pm0.2$).

To investigate whether the [³H]-InsP$_x$ effect of ADP was mediated by P2Y$_1$ receptors, the antagonists A3P5P (Boyer *et al.*, 1996) and MRS 2179 (Boyer *et al.*, 1998) were used. The inclusion of 100 $\mu$M A3P5P led to a slight reduction in the effect of ADP at concentrations above 10 $\mu$M, as illustrated in Figure 3a. At the maximal concentration of ADP the response in the presence of A3P5P was reduced by $17.3\pm2.5\%$ ($P<0.05$, data pooled across three experiments). As shown in Figure 3b, the inclusion of MRS 2179 had no effect on the concentration-response curve to ADP (the response to 300 $\mu$M ADP was reduced by $4.8\pm7.3\%$, $n=3$).

To explore the possibility that the ADP effect was due to conversion to ATP in the extracellular compartment, experiments with hexokinase were conducted. ADP stock was treated with hexokinase to eliminate contaminating ATP, and an additional 3 units of enzyme were included in each well to prevent conversion of ADP to ATP during the stimulation period. The effect was to significantly reduce the effectiveness of ADP (Figure 4); over three experiments the response to 100 $\mu$M ADP was reduced by $55.7\pm9.8\%$ ($P<0.001$, Bonferroni's post-test). This result indicated that interconversion of ADP to ATP was contributing to the

observed ADP response. HPLC analysis was performed to further elucidate the changes occurring in the nucleotide composition of the medium. UTP, ITP and 2-MeSADP were all found to be stable for the 20 min stimulation period. The relative amounts of nucleotides recovered after incubating cells for 20 min with 300 $\mu$M ATP are shown in Table 1. The ATP stock was determined to be essentially free of contaminants. The stock ADP solution used was found to be contaminated with 3% ATP and 11% AMP (no adenosine was detected). As shown in Table 1 ADP was actively converted to AMP and ATP during the assay period. The inclusion of hexokinase in the incubation served to exacerbate the decline in ADP concentration, with a coincident decrease in ATP levels as expected. The overall effect was to channel ADP into AMP (Table 1).

The enzyme adenylate kinase catalyses the reaction $2.\text{ADP}\leftrightarrow\text{AMP}+\text{ATP}$ and could account for the observed interconversions. We therefore conducted experiments with the adenylate kinase inhibitor, Ap$_5$A (Lienhard & Secemski, 1973) to examine the contribution of this enzyme to ADP-stimulated [³H]-InsP$_x$ accumulation. As shown in Table 1, incubating cells with 300 $\mu$M ADP in the presence of a maximally effective concentration of Ap$_5$A (300 $\mu$M), largely inhibited the interconversion of ADP. Although some ATP and AMP were still present, increasing the Ap$_5$A concentration to 1 mM did not further protect ADP concentrations. ADP-stimulated accumulation of [³H]-InsP$_x$ was significantly reduced by inclusion of 300 $\mu$M Ap$_5$A as shown in Figure 5. At concentrations of ADP below 30 $\mu$M, Ap$_5$A effectively blocked the accumulation of [³H]-InsP$_x$. At higher concentrations the response was reduced (at 100 $\mu$M ADP by $69.5\pm4.9\%$ and 300 $\mu$M by $32.8\pm9.2\%$; $n=3$).
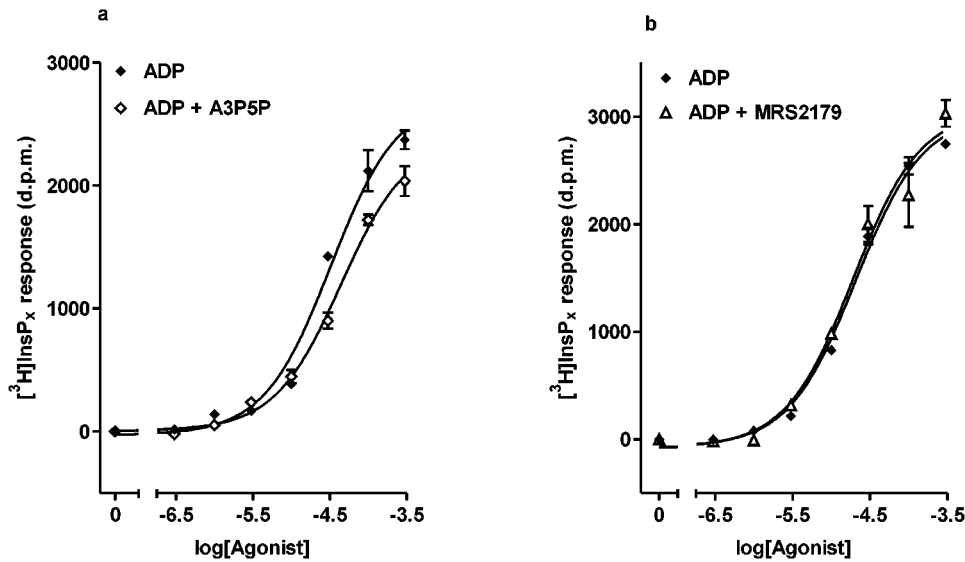
**Figure 3** Effects of the P2Y$_1$ antagonists, A3P5P and MRS 2179 on the inositol (poly)phosphate response to ADP. Dose–response curves to increasing concentrations of ADP were constructed in the presence and absence of 100 $\mu$M A3P5P (a) or 100 $\mu$M MRS 2179 (b). Results, shown as d.p.m. after subtraction of unstimulated controls, are from a single representative experiment, with stimulations performed in triplicate. Data pooled from three separate experiments are reported in the text.
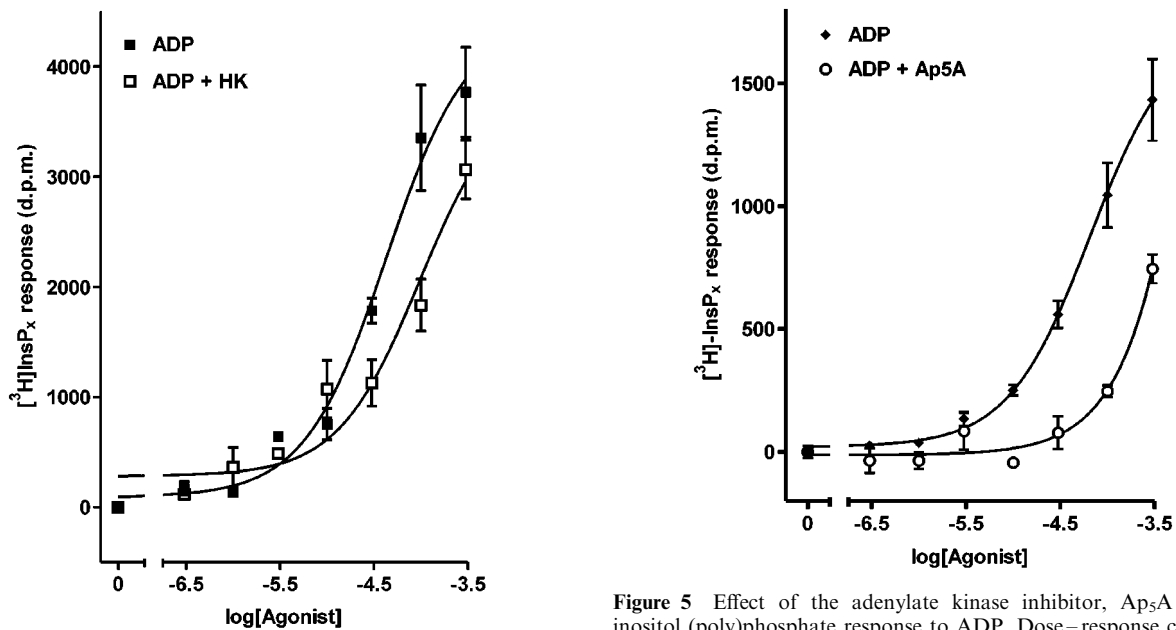


**Figure 4** Effect of hexokinase-treatment on the inositol (poly)phosphate response to ADP. Dose–response curves to increasing concentrations of untreated, and hexokinase-treated ADP. Hexokinase was also included in the assay (3 units well$^{-1}$) to counteract conversion of ADP to ATP during the 20 min stimulation period. Results, shown as d.p.m. after subtraction of unstimulated controls, are from a single representative experiment, with stimulations performed in triplicate. Data pooled from three separate experiments are reported in the text.



**Figure 5** Effect of the adenylate kinase inhibitor, Ap$_5$A on the inositol (poly)phosphate response to ADP. Dose–response curves to increasing concentrations of ADP were constructed in the presence and absence of 300 $\mu$M Ap$_5$A. Results, shown as d.p.m. after subtraction of unstimulated controls, are from a single representative experiment, with stimulations performed in triplicate. Data pooled from three separate experiments are reported in the text.

The adenylate kinase-catalysed breakdown of ADP produces AMP in addition to ATP (Table 1). We therefore investigated the contribution of AMP to the ADP response through use of a P1 antagonist. The inclusion of the non-selective P1 antagonist, CGS 15943 (1 $\mu$M; Alexander *et al.*, 1996) in the assay led to a small, although not significant, increase in the response at 300 $\mu$M ADP (111.1$\pm$15%, $P>0.05$, $n=3$; data not shown). The apparent EC$_{50}$ of ADP was not affected by the presence of CGS 15943 (logEC$_{50}$ for ADP, $-4.5\pm0.3$; logEC$_{50}$ for ADP$+1$ $\mu$M CGS 15943, 4.4$\pm$0.2).

**Table 1** Conversion of ADP and ATP by cultured hepatocytes

| Agonist (300 $\mu$M) | ATP ($\mu$M) | ADP ($\mu$M) | AMP ($\mu$M) | Ado ($\mu$M) |
|---|---|---|---|---|
| ATP | 343±4 | 33±4 | 15±3 | 14±1 |
| ADP | 109±1 | 101±4 | 141±2 | 27±1 |
| ADP+HK | 0* | 82±9 | 243±8 | 29±16 |
| ADP+Ap₅A | 32±4 | 245±33 | 87±16 | 21±1 |

Hepatocytes were incubated with ATP, and ADP (300 $\mu$M) either alone or in the presence of hexokinase (HK; 6 units ml$^{-1}$) or the adenylate kinase inhibitor, Ap₅A (300 $\mu$M) for 20 min, and the supernatants analysed by HPLC to determine nucleotide concentrations. Data are mean±s.e.-mean pooled from three separate experiments. *Level below detectable limit.

## Discussion

Rat hepatocytes are known to express multiple P2Y receptors mediating the effects of extracellular nucleotides (Dixon *et al.*, 1990; 2000; Keppens, 1993). In a previous report we concluded that rat hepatocytes express both P2Y₁ and P2Y₂ receptors, and that these receptors mediate the effects of ADP and ATP/UTP respectively (Dixon *et al.*, 2000). In the absence of selective antagonists, the conclusion that functional P2Y₂ receptors are expressed was based upon similarities in [Ca$^{2+}$]$_i$ transients induced by ATP and UTP (Dixon *et al.*, 2000; Keppens *et al.*, 1992), and on the sensitivity of the responses to suramin (Dixon *et al.*, 2000). The similarity in the inositol phosphate responses reported here to UTP and ATP is consistent with the expression of P2Y₂ receptors, at which ATP and UTP are equipotent. However, rat P2Y₄ receptors are also activated equally by ATP and UTP (Bogdanov *et al.*, 1998; Webb *et al.*, 1998), unlike the human homologue which displays a marked preference for UTP (Communi *et al.*, 1995; Nguyen *et al.*, 1995). The reduced response to ITP reported here compared with ATP and UTP argues against the sole involvement of P2Y₄ receptors, as all three of these nucleotides are full and equipotent agonists at this receptor (Bogdanov *et al.*, 1998). The results presented here then are consistent with the earlier conclusion that P2Y₂ receptors mediate the effects of UTP and ATP in rat hepatocytes, although a contribution to the ATP/UTP response by the P2Y₄ receptor cannot be completely discounted. The definitive test of these conclusions awaits the advent of selective ligands/antagonists for P2Y₂ and P2Y₄ receptors.

2-MeSADP stimulates a [Ca$^{2+}$]$_i$ response in rat hepatocytes which is blocked by the P2Y₁ antagonist, A3P5P (Dixon, 2000). However, as demonstrated here this nucleotide evokes only a small rise in InsP$_x$. Similarly, ADP$\beta$S, a full agonist at the human P2Y₁ receptor (Leon *et al.*, 1997), failed to evoke a [³H]-InsP$_x$ response but does elicit a rise in [Ca$^{2+}$]$_i$ in rat hepatocytes (Keppens *et al.*, 1993). 2-MeSATP also stimulated [Ca$^{2+}$]$_i$ transients which are inhibited by A3P5P (Dixon, 2000), yet this nucleotide failed to increase Ins(1,4,5)P₃ levels (Keppens & De Wulf, 1995). The rat hepatocyte P2Y₁ receptor then appears not to be coupled to increases in Ins(1,4,5)P₃. In contrast, the cloned rat P2Y₁ receptor has been shown to couple to inositol phosphate accumulation when transfected into 1321N1 human astrocytoma cells and C6 rat glioma cells (Schachter *et al.*, 1997). The reason for

this disparity is not clear; it may reflect differences in cloned and native systems, in terms of receptor number or signal transduction systems. Inositol phosphate-independent increases in [Ca$^{2+}$]$_i$ have also been noted in rat brain capillary endothelial cells in response to ADP and 2-MeSATP (Frelin *et al.*, 1993; Vigne *et al.*, 1994; Albert *et al.*, 1997).

Previous work has indicated that ADP acts at the same receptor as 2-MeSADP on rat hepatocytes (Dixon, 2000). Therefore the observation that ADP stimulation led to [³H]-InsP$_x$ accumulation, whilst 2-MeSADP evoked only a small response was surprising. The P2Y₁ receptor antagonist, A3P5P had little effect on the dose-response curve to ADP. The reported pK$_B$ for this antagonist at the human P2Y₁ receptor is ~6 (Boyer *et al.*, 1996). Therefore the minimal shift in the ADP curve seen here in the presence of 100 $\mu$M A3P5P is inconsistent with the major part of the [³H]InsP$_x$ response being mediated by P2Y₁ receptors. Furthermore the highly selective P2Y₁ receptor antagonist, MRS 2179 (pK$_B$ ~7; Boyer *et al.*, 1998) had no effect on the ADP-mediated [³H]-InsP$_x$ accumulation. It appears from these results that the response to ADP is not mediated by P2Y₁ receptors.

HPLC analysis revealed that ADP in the medium was actively converted to AMP and ATP so that similar amounts of all three nucleotides were recovered following the 20 min stimulation period. Adenylate kinase catalyzes the reaction $2.ADP \leftrightarrow ATP + AMP$ and could therefore account for the observed nucleotide conversions. This enzyme is inhibited by Ap₅A (Lienhard & Secemski, 1973) and consistent with its presence on rat hepatocytes, Ap₅A protected ADP from conversion to AMP and ATP. (Following 20 min stimulation with 300 $\mu$M ADP ~65% was preserved, compared with 27% in the absence of Ap₅A.) The accumulation of [³H]-InsP$_x$ measured in response to ADP was therefore likely to be dependent upon its conversion to AMP and ATP. Inclusion of the non-selective P1 antagonist, CGS 15943 had no effect on ADP-stimulated [³H]-InsP$_x$ accumulation. This indicates that AMP formed through the action of adenylate kinase does not contribute to the ADP response. However, consistent with the proposal that the ADP response is dependent on the action of adenylate kinase, there was a coincident decrease in [³H]-InsP$_x$ accumulation in response to ADP when Ap₅A was included in the assay. At less than 30 $\mu$M ADP, the response was effectively blocked by Ap₅A. The residual response to ADP at concentrations over 30 $\mu$M in the presence of Ap₅A can be accounted for by the small amounts of ATP formed (~30 $\mu$M ATP when 300 $\mu$M ADP applied). It is clear then from these data that most, if not all, of the ADP response is secondary to its conversion to ATP by an ecto-adenylate kinase and action at a P2Y₂ receptor. Although traditionally regarded as an intracellular enzyme, ecto-adenylate kinase activities have been reported on human umbilical vein endothelial cells (Yegutkin *et al.*, 2001) and human airway surfaces (Donaldson *et al.*, 2002). The presence of adenylate kinase will play an important role in determining the nucleotide composition of the extracellular milieu, and will therefore act as a regulator of hepatocyte function.

Another enzyme capable of extracellular conversion of ADP to ATP is ecto-nucleoside diphosphokinase (Lazarowski *et al.*, 1997). However, if this enzyme were responsible for ATP formation in the present study, AMP would not be generated as reported. In addition the [³H]-InsPx response to

ADP and the formation of ATP would not be inhibited by Ap$_5$A. We conclude, therefore, that diphosphokinase activity is not responsible for the hepatocyte response to ADP.

Hexokinase treatment is used to convert ATP formed during the incubation back into ADP. The reduced effect of ADP seen in the presence of hexokinase therefore confirms the significance of formation of ATP in mediating this response. However, removing ATP with hexokinase did not have as great an effect as may be expected. It is possible that the levels of nucleotides monitored in the bulk phase by HPLC may not mirror those at the cell surface, where both ecto-enzyme and P2Y receptors are located. However, it also remains a possibility that ADP acts in part at a hitherto uncloned receptor which is not stimulated by ADP$\beta$S or 2MeSADP.

In conclusion, we have provided evidence that the ADP-stimulated inositol (poly)phosphate formation in rat hepato-cytes is secondary to its conversion to ATP by adenylate kinase. It is striking that while ADP was very rapidly metabolized by cultured hepatocytes, ATP was relatively stable. This indicates that these cells have little ecto-nucleotidase activity, but a very active ecto-adenylate kinase. The consequence will be to limit stimulation of P2Y$_1$ receptors, whilst enhancing P2Y$_2$ activation, in response to released native nucleotides. AMP, generated along with ATP by adenylate kinase, will act at P1 receptors on hepatocytes which are coupled to increases in cAMP (Okajima *et al.*, 1987), further potentiating the response to ATP; it has previously been shown that elevated cAMP levels enhance the hepatocyte [Ca$^{2+}$]$_i$ response to ATP (Green *et al.*, 1994).

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