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Regulation of rat hepatocyte function by P2Y receptors: focus on control of glycogen phosphorylase and cyclic AMP by 2-MeSADP

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Running title page.

Running Title: P2Y receptors and hepatocytes.

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Abbreviations: A3P5P, adenosine-3′–phosphate-5′–phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high pressure liquid chromatography; IBMX, 3-isobutyl-1-methylxanthine; 2-MeSADP, 2-methylthioADP; RT-PCR, reverse transcriptase-polymerase chain reaction; WME, William's medium E.

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Abstract

Hepatocyte function is regulated by several P2Y receptor subtypes. Here we report that 2-MeSADP, an agonist at $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors, potently (threshold 30nM) stimulates glycogen phosphorylase in freshly-isolated rat hepatocytes. Antagonism by MRS 2179 confirms that this response is mediated by $P2Y_1$ receptors. In addition, in these cells both 2-MeSADP and UTP inhibited glucagon-stimulated cyclic AMP accumulation. This inhibitory effect of 2-MeSADP was not reversed by the $P2Y_1$ antagonists, adenosine-3′–phosphate-5′–phosphate (A3P5P) or MRS 2179, both in the range 1-300 μM, indicating that it was not mediated by $P2Y_1$ receptors. This contrasts with the increase in cytosolic free Ca^{2+} concentration $[Ca^{2+}]_c$ induced by 2-MeSADP, which has shown to be inhibited by A3P5P. Pertussis toxin abolished the inhibitory effect of both UTP and 2-MeSADP. Following culture of cells for 48 hours, the ability of 2-MeSADP to inhibit cyclic AMP accumulation was greatly diminished. Reverse transcriptase-PCR analysis revealed that during this culture period there was a decline in the ability to detect transcripts for $P2Y_{12}$ and $P2Y_{13}$ receptors, both of which are activated by 2-MeSADP and negatively coupled to adenylyl cyclase. However, in freshly-isolated cells, the $P2Y_{12}$ and $P2Y_{13}$ receptor antagonist, AR-C67085 (10 nM-300 μM) did not alter the ability of 2-MeSADP to inhibit glucagon-stimulated cyclic AMP accumulation. We conclude that 2-MeSADP regulates rat hepatocyte glycogen phosphorylase by acting on $P2Y_1$ receptors coupled to raised $[Ca^{2+}]_c$, and by inhibiting cyclic AMP levels by an unknown G_i-coupled receptor subtype, distinct from $P2Y_1$, $P2Y_{12}$ or $P2Y_{13}$ receptors.

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INTRODUCTION

Glycogen phosphorylase, the rate-controlling enzyme in hepatic glycogenolysis, is activated by both increases in cyclic AMP and cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$), resulting in a net output of glucose to supply extra-hepatic tissues, crucially the brain, when blood glucose levels drop. Extracellular nucleotides play a well-established role in the regulation of this key function in rat hepatocytes through the activation of P2Y receptors (Okajima et al., 1987; Keppens, 1993; Keppens et al., 1992, 1993), a family of G-protein-coupled receptors responding to the native nucleotides ADP, ATP, UDP, UTP and UDP-glucose (Boarder and Hourani, 1998; Abbracchio et al., 2003). Curiously, stimulation of P2Y receptors on rat hepatocytes leads to both increases in $[Ca^{2+}]_c$ and inhibition of adenylyl cyclase; these responses will stimulate and limit activation of glycogen phosphorylase respectively (Okajima et al., 1987; Dixon et al., 1990; 1995; 2000; Keppens, 1993; Keppens et al., 1992; 1993; Edgecombe et al., 1999). The mechanism underlying the regulation of glycogen phosphorylase by cyclic AMP is well established, with increased cyclic AMP levels leading to phosphorylation and activation of the enzyme, with dephosphorylation by protein phosphatase 1 as cyclic AMP levels fall. Little is understood of P2Y receptor regulation of cyclic AMP in hepatocytes, despite its significance in the control of glycogen phosphorylase. An understanding of the nature of the P2Y receptors present on hepatocytes and their influence on cell function is of considerable physiological relevance since it is known that native nucleotides reach micromolar concentrations in the extracellular compartment of the liver (Chari et al., 1996). Furthermore release of ATP by hepatocytes and activation of P2Y receptors on neighbouring cells has been shown to play a role in intercellular communication in the liver (Schlosser et al., 1996; Wang et al., 1996).

In common with many other cell types, rat hepatocytes are known to express multiple P2Y subtypes, although these have not been conclusively identified (Dixon et al., 1990; 1995; 2000; 2003; Keppens 1993). Reverse transcriptase-polymerase chain reaction (RT-PCR) has

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demonstrated the presence in primary rat hepatocytes of mRNA transcripts encoding all 4 of the cloned rat P2Y subtypes investigated ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$; Dixon et al., 2000). These receptors are coupled to increases in $[Ca^{2+}]_c$. We have previously argued that rat hepatocytes express functional $P2Y_1$ and $P2Y_2$ receptors mediating the effects of ADP ($P2Y_1$) and ATP and UTP ($P2Y_2$), with a possible contribution from $P2Y_4$ (Dixon et al., 2000;2003). The $P2Y_6$ receptor is apparently not expressed as a functional receptor, since UDP, which is the most potent agonist at this receptor (Nicholas et al., 1996), failed to elicit a $[Ca^{2+}]_c$ response in rat hepatocytes (Dixon et al., 2000).

The most recently characterised members of the P2Y receptor family are coupled to changes in cyclic AMP levels. Thus, the $P2Y_{11}$ receptor is coupled positively through G_s to adenylyl cyclase, in addition to the phosphoinositide pathway (Communi et al., 1999; Zambon et al., 2001). $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors are coupled negatively to adenylyl cyclase through G_i (Abbracchio et al., 2003; Communi et al., 2001; Hollopeter et al., 2001; Zhang et al., 2001; 2002).

2-MeSADP is a partially selective P2Y agonist, acting at $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors (Communi et al., 2001; Hollopeter et al., 2001; Zhang et al., 2002). Here we demonstrate that 2-MeSADP both stimulates glycogen phosphorylase and inhibits cyclic AMP in rat hepatocytes, and have investigated the hypothesis that this occurs by action at distinct P2Y receptors.

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METHODS

Cell preparation and stimulation

Hepatocytes were isolated from fed, male Wistar-strain rats (150-250g) 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 30ml min^{-1} throughout. The cells were harvested and re-suspended in Hepes-buffered medium at the appropriate density (116 mM NaCl; 5.6 mM KCl; 0.8 mM $MgSO_4$; 1.1 mM KH_2PO_4; 4.8 mM $NaHCO_3$; 11 mM glucose; 1.8 mM $CaCl_2$, 20 mM Hepes; 2% BSA w v^{-1}; pH 7.4).

Cell culture

Isolated cells were cultured for 24 hours at a density of 1 x 10^5 cells $well^{-1}$ in 24-well plates in William's medium E with 10% FCS, 50 iu ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 50 μg ml^{-1} gentamicin, in a volume of 0.5 ml. Cells were then cultured for a further 24 hours without FCS. For experiments investigating the effect of pertussis toxin (100 ng ml^{-1}), cells were cultured for a total of 24 hours with the toxin added, where appropriate, at the same time that the cells were plated out.

Cyclic AMP measurement

Cultured cells, or freshly-isolated cells re-suspended at 2 x 10^6 cells ml^{-1}, were treated with 300 μM IBMX and P2Y antagonists where applicable, for 10 min prior to agonist addition. Aliquots of freshly-isolated cells were dispensed into a 24-well plate (0.25 ml per well) and shaken at 37°C. Cells were stimulated by co-addition of nucleotide and glucagon (10 nM final concentration) for 3 min, and incubations terminated by addition of TCA. Samples were left on ice for 60 min, extracted with a 1:1 mixture of tri-n-octylamine and 1,1,2-trichloro-

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trifluoroethane, neutralized with 250 mM $NaHCO_3$, and assayed for cyclic AMP content using the protein binding assay of Brown et al. (1971).

Glycogen phosphorylase assay

Cells were re-suspended at 4 x 10^6 cells ml^{-1} and treated with MRS 2179 for 10 min where applicable. Aliquots of 0.25 ml were dispensed into each well of a 24-well plate and shaken at 37°C and cells stimulated with 2-MeSADP for 2 min. Incubations were terminated by removing 100 µl of cell suspension and adding to 100 µl extraction buffer (400 mM NaF; 80 mM EDTA; 2% (w/v) glycogen; 200 mM glycyl glycine; pH 7.4) and freezing immediately in liquid nitrogen. Cell extracts were subjected to three cycles of freeze-thawing and centrifuged at 10,000 g for 1 min at 4°C. A 100 µl portion of supernatant was incubated with 20 µl of assay buffer (600 mM glucose-1-phosphate; 1.8 M NaF; 6 mM caffeine; 12% (w/v) glycogen; 29.6 KBq ml^{-1} [^{14}C]glucose-1-phosphate; pH 6.1) for 40 min at 30°C. The reaction was terminated by spotting 100 µl of the assay mixture onto filter papers that were washed in 66% EtOH for 1.5 hours, briefly soaked in acetone and dried before being counted for radioactivity.

Calculation of antagonist potency

An estimate of the pA_2 value for MRS 2179 was calculated with a rearrangement of the Schild equation: $pA_2 = -\log_{10}([B]/CR-1)$, where [B] is the concentration of agonist tested and CR is the concentration ratio of curve mid-point in the presence and absence of antagonist. In the absence of a full Schild analysis this is referred to as an apparent pA_2.

High pressure liquid chromatography (HPLC) analysis

HPLC analysis was used to determine whether 2-MeSADP and antagonists were broken down during the incubation period. An Alltech SAX column was eluted with a gradient running from A: 0.05M to B: 1 M K_2HPO_4 of 100%A for 10 min, to 20% B 10 -12.5 min, to 70% B 12.5 - 25 min.

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Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was prepared from freshly isolated hepatocytes (~6 x 10^6 cells) using Trizol according to the manufacturer's instructions, and was treated with RNase-free DNase I (200 U) for 30 min at 37°C and then re-extracted with Trizol. Isolated hepatocytes were also cultured (3 x 10^6 cells/9 cm dish) as described above for 24 hours, and then for a further 24 hours in the absence of FCS and RNA extracted as described above. First strand cDNA was prepared from 2.5 μg RNA using random hexamers and Superscript II reverse transcriptase in a 20 μl reaction volume, according to the manufacturer's instructions. PCR reactions for the rat $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors employed the primers described previously (Dixon et al., 2000). Reactions for the $P2Y_{12}$ and $P2Y_{13}$ receptors included the following primer pairs designed to amplify partial cDNAs from each sequence ($P2Y_{12}$: 5'-ATCTGGGCCTTCATGTTCCTGCT GTC-3', 5'-CTTTCTTCTTATTTGCCCCG GATGTTGAG-3', 532 bp; $P2Y_{13}$ 5'-TACCATACACCCACAGTCAAACCACC-3', 5'-GGGCATAATCTTTTCCGTACAC GAG-3', 405 bp). The rat $P2Y_{12}$ primers were designed from the published sequence (Simon et al., 2002), and the rat $P2Y_{13}$ primers were designed from a rat expressed sequence tag (accession number BF390809) that when translated shared 80 % amino acid identity with the corresponding region of the human protein. Amplifications of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were also performed using the following primers (5'-ACCACAGTCCATGCCATCAC-3', 5'-TCCACCACCCTGTTGCTGTA-3', 415 bp).

PCR reactions were performed using 1.25 % (v v^{-1}) of each first strand cDNA reaction and 7.5 pmol of each subtype-specific forward and reverse primer, with 2.5 units of Biotaq in a 25 μl amplification volume containing 1.5 mM $MgCl_2$, according to the manufacturer's instructions. Amplification conditions were 30 s at 94°C; 30 s at 65°C, 45 s at 72°C for 30 cycles. Reverse transcriptions were also performed in the absence of the enzyme as a control for contaminating DNA, and control amplifications were performed in the absence of added template. Amplicons (5

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μl for GAPDH, $P2Y_1$, $P2Y_2$ and 10 μl for $P2Y_4$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$) were subjected to gel electrophoresis on a 2% (w v^{-1}) agarose gel.

Statistical analysis

Glycogen phosphorylase and cyclic AMP data presented in figures are representative of 3 experiments from different hepatocyte preparations and expressed as milliunits of enzyme activity or pmoles of cyclic AMP $well^{-1}$. All data presented in the text were pooled across 3 experiments each in triplicate, and cyclic AMP data normalized to the glucagon response after subtraction of basal values. Statistical analysis was by one-way ANOVA with Bonferroni's post-test, or Student's t-test where indicated.

Materials

Cell culture medium was from GIBCO BRL (Paisley,U.K.). [^{3}H]cyclic AMP and [^{14}C]glucose-1-phosphate were purchased from Amersham Pharmacia Biotech (Little Chalfont, Bucks, U.K.). Collagenase was from Boehringer (Lewes, U.K.). 2-MeSADP, A3P5P, MRS 2179 (N^6-methyl 2'-deoxyadenosine 3',5'-bisphosphate), UTP, pertussis toxin and all other chemicals were from Sigma-Aldrich (Poole, U.K.). AR-C67085 (2-propylthio-β,γ-dichloromethylene-D-ATP) was a kind gift from AstraZeneca R & D Charnwood (Loughborough, U.K.). Biotaq was purchased from Bioline (Edgeware, UK) and all other molecular biology reagents were obtained from Invitrogen Life Technologies (Paisley, UK).

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RESULTS

Activation of glycogen phosphorylase by 2-MeSADP in freshly-isolated rat hepatocytes

Figure 1 shows the concentration-response curve for activation of glycogen phosphorylase by 2-MeSADP in the presence and absence of the highly-selective $P2Y_1$ antagonist, MRS 2179 (Boyer et al., 1998). 2-MeSADP potently stimulates glycogen phosphorylase with responses starting at 30 nM. In all experiments the curve was biphasic; an initial plateau (~80% of maximal response) formed at 10-30 μM 2-MeSADP, followed by a small further rise at higher concentrations. The mean EC_{50} for the major, high potency component of the response was 121 nM (pEC_{50} = -6.92 ± 0.02: data pooled across 3 separate experiments, each in triplicate). In the presence of 100 μM MRS 2179 there was a substantial shift of the concentration-response curve to the right, with an EC_{50} of 81 μM (pEC_{50} = -4.17 ± 0.21 μM: data pooled across 3 experiments). The apparent pA_2 for MRS 2179, calculated only from the initial major phase of the concentration-response curve for 2MeSADP (i.e. up to 100 μM 2MeSADP), was 6.82 (data pooled from 3 experiments).

Inhibition of glucagon-stimulated cyclic AMP accumulation by 2-MeSADP and UTP in freshly-isolated rat hepatocytes

As expected, glucagon (10 nM) in the presence of the phosphodiesterase inhibitor IBMX (300 μM), stimulated cyclic AMP accumulation from basal values of 7.8 ± 0.6 to 40.5 ± 0.8 pmoles cyclic AMP per well (n=3 separate experiments). Stimulation of cells with 2-MeSADP led to a decrease in cyclic AMP levels stimulated by glucagon (Figure 2a). The threshold concentration of 2-MeSADP for this inhibitory response was greater than 30 μM; application of 10 or 30 μM 2-MeSADP had no significant effect. Application of 100 μM 2-MeSADP reduced the glucagon-stimulated cyclic AMP level to 27.2 ± 1.1 pmoles per well. Increasing the 2-MeSADP concentration to 300 μM led to a further decrease in cyclic AMP concentrations to 15.4

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± 0.4 pmoles per well (Figure 2a). This level of inhibition was found to be maximal; application of 1 mM 2-MeSADP had no additional effect (data not shown).

UTP was found to inhibit glucagon-stimulated cyclic AMP levels in freshly-isolated hepatocytes to a similar extent (Figure 2a). Thus, after subtraction of basal values, a maximal concentration of UTP (300 μM) reduced the glucagon-stimulated cyclic AMP level by 61.2 ± 1.6% compared with 61.9 ± 0.3% in response to 300 μM 2-MeSADP (n=3 separate experiments). When applied alone to cells neither 2-MeSADP nor UTP (300 μM) had any significant effect on the basal level of cyclic AMP (data not shown).

Breakdown of UTP and 2-MeSADP

When cells were incubated with 300 μM UTP for 3 min and the medium analyzed by HPLC, only 95.0 ± 8.7 μM UTP was recovered. The majority of nucleotide (219.0 ± 6.0 μM) was recovered as UDP. Similarly, following incubation of cells for 3 min with 300 μM 2-MeSADP, 132.9 ± 9.5 μM was recovered. Two additional peaks were identified with retention times of 8.7 and 20.7 min compared with 27.9 min for 2-MeSADP, consistent with the 2-methylthio-substituted derivatives of adenosine and AMP respectively. These two peaks accounted for 29.4 ± 2.4 % and 26.7 ± 0.6 % of the added nucleotide concentration (data not shown).

The products of 2-MeSADP degradation could conceivably act on P1 receptors present on rat hepatocytes and therefore influence the observed response. The P1 receptor present on these cells has been characterized as an A_{2B} receptor coupled to increases in cyclic AMP (Yasuda et al., 2003). Stimulation of this receptor would therefore counteract the inhibitory effect of 2-MeSADP on cyclic AMP generation. The inclusion of 300 μM IBMX should block P1 receptor activation as this compound is a general low potency P1 antagonist (Fredholm et al., 1994). However, to eliminate this possibility we stimulated cells with 2-MeSADP in the additional presence of the non-selective P1 antagonist CGS 15943 (Alexander et al., 1996). This treatment had no significant effect on the ability of 2-MeSADP to inhibit glucagon-stimulated cyclic AMP levels.

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Thus in response to 100 μM and 300 μM 2-MeSADP, glucagon-stimulated cyclic AMP levels were reduced by 28.0 ± 7.9% and 71.8 ± 1.7%, compared with 24.2 ± 9.9% and 70.8 ± 3.9% in the presence of 5 μM CGS 15943 (n =3; data not shown). In addition, inclusion of CGS 15943 did not increase the potency of 2-MeSADP; 30 μM 2-MeSADP had no significant effect in the presence or absence of CGS 15943 (data not shown).

Effect of culturing rat hepatocytes on the response to 2-MeSADP and UTP

Hepatocytes were cultured for 48 hours and the effects of 2-MeSADP and UTP examined and compared with the effects in freshly-isolated cells from the same preparation. As illustrated in Figure 2b, the inhibitory effect of 300 μM 2-MeSADP on glucagon-stimulated cyclic AMP levels was reduced significantly from 61.9 ± 0.3% in freshly-isolated cells to 22.9 ± 6.2% following culture (P<0.01, Student's t-test). In contrast, the inhibitory effect of 300 μM UTP was significantly increased from 61.2 ± 1.6% to 88.7 ± 4.5% after 48 hours in culture (P<0.01, Student's t-test).

To investigate the effects of pertussis toxin, the culture period was reduced to 24 hours, at which time an inhibitory effect of 2-MeSADP was still apparent. Expressed as pmoles cyclic AMP per well (mean ± s.e.), glucagon (10 nM) stimulation (control-deducted) with no pertussis toxin treatment was 9.7 ± 0.5, which was reduced by 2-MeSADP (300 μM) to 7.0 ± 1.2 and by UTP (300 μM) to 1.1 ± 0.1. After pertussis toxin, the equivalent figures were: glucagon, 11.3 ± 0.5; glucagon plus 2-MeSADP, 10.44 ± 0.31; glucagon plus UTP, 9.82 ± 1.34 pmoles cyclic AMP per well. These results show that pertussis toxin pre-treatment removes the majority of the inhibitory cyclic AMP response to 2MeSADP and to UTP. HPLC analysis revealed that during the 3 min incubation with cultured cells 2-MeSADP and UTP were not degraded (data not shown).

Expression of mRNA for P2Y receptor subtypes

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RT-PCR studies were undertaken to compare the expression of the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ receptor transcripts in RNA isolated from freshly-isolated hepatocytes and cells from the same preparations cultured for 48 hours. Products for rat $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ receptor mRNAs were detected when cDNA derived from freshly-isolated hepatocytes was subjected to 30 PCR cycles in reactions that included reverse transcriptase, but not when it was omitted (Figure 3).

Amplifications using RNA isolated from cultured hepatocytes revealed an altered expression pattern for P2Y receptor transcripts, with a stronger signal from $P2Y_4$ and $P2Y_6$ amplicons when compared to freshly-isolated samples. Conversely, transcripts for the $P2Y_{12}$ receptor were barely detectable, and $P2Y_{13}$ receptor transcripts were absent after 30 cycles of PCR (Figure 3). However, the intensities of bands for $P2Y_1$, $P2Y_2$ and the housekeeping gene, GAPDH were indistinguishable between freshly-isolated and cultured preparations, indicating that the altered expression pattern is likely to be a reflection of true differences in P2Y receptor transcript levels, rather than differences resulting from sample preparation. No PCR products were detected when reactions were performed in the absence of added template (data not shown).

Effects of $P2Y_1$ antagonists on 2-MeSADP-stimulated cyclic AMP responses

In a series of experiments we investigated the effect of two $P2Y_1$ antagonists, A3P5P (Boyer et al., 1996) and MRS 2179 (Boyer et al., 1998). Figures 4a and b show that MRS 2179, in the concentration range 1-300μM, did not reverse the inhibition of glucagon-stimulated cyclic AMP levels induced by either 100 μM or 300 μM 2-MeSADP. Data pooled across 3 experiments with 300 μM 2-MeSADP showed no significant effect of 1-300 μM MRS 2179 ($P>0.05$, one way ANOVA). However, at the sub-maximal concentration of 2-MeSADP (100 μM) there was a tendency for MRS 2179 to further reduce cyclic AMP levels (Figure 4a) which was significant when data were pooled from 3 experiments ($P<0.001$, one way ANOVA). This effect is seen in the absence of stimulation with nucleotide agonists: the application of 300 μM MRS 2179 to rat

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hepatocytes in the absence of 2-MeSADP reduced glucagon-stimulated cyclic AMP levels by 31.2 ± 11.4 % (n = 3 separate experiments).

The less-selective $P2Y_1$ antagonist A3P5P, gave essentially the same results as MRS 2179. Data pooled from 3 experiments revealed that A3P5P (1-300 μM) had no significant effect on the inhibition of glucagon-stimulated cyclic AMP levels induced by 300 μM 2-MeSADP ($P>0.05$, one-way ANOVA; data not shown). Again at the sub-maximal concentration of 2-MeSADP (100 μM), there was a significant effect of A3P5P to further reduce cyclic AMP levels ($P > 0.05$; one-way ANOVA). Application of 300 μM A3P5P alone led to a reduction of glucagon-stimulated cyclic AMP levels by 34.8 ± 3.0 % (n = 3 separate experiments).

Effects of AR-C67085 on 2-MeSADP-stimulated cyclic AMP responses

As 2-MeSADP is also a highly potent agonist at the rat $P2Y_{12}$ receptor, we investigated the effect of the $P2Y_{12}$ receptor antagonist, AR-C67085 (Humphries et al., 1995a; 1995b). Figures 5a and b show the results from a typical experiment in which increasing concentrations of AR-C67085 (10 nM-1 μM) did not affect the inhibition of glucagon-stimulated cyclic AMP levels by a sub-maximal (100 μM) or maximal dose (300 μM) of 2-MeSADP. Data pooled from 3 experiments revealed that AR-C67085 had no significant effect at either concentration of 2-MeSADP ($P>0.05$, one-way ANOVA). Unlike MRS 2179 and A3P5P, application of AR-C67085 alone had no significant effect on glucagon-stimulated cyclic AMP levels; response to 10 nM glucagon in the presence of 1 μM AR-C67085 was 100.3 ± 13.3% of the response to glucagon alone.

AR-C67085 has recently been described as a weaker potency antagonist at the $P2Y_{13}$ receptor (Marteau et al., 2003). Increasing the AR-C67085 concentration to 300 μM did not lead to inhibition of the 2-MeSADP-mediated attenuation of cyclic AMP levels (data not shown).

AR-C67085 is also a potent agonist at the human $P2Y_{11}$ receptor, with an EC_{50} for cyclic AMP accumulation in transfected cells of 1.5 μM (Communi et al., 1999). When applied to cells

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at concentrations up to 300 μM, AR-C67085 had no effect on cyclic AMP levels. Thus, in the presence of 300 μM AR-C67085, the cyclic AMP concentration was 15.0 ± 2.3 compared with basal values of 13.3 ± 2.3 pmoles cyclic AMP per10^6 cells (n=3 separate experiments; $P>0.05$, Student's t-test). In a parallel series of experiments of AR-C67085 increased cyclic AMP levels in 1321N1 cells heterologously expressing $P2Y_{11}$ receptors (unpublished data).

HPLC analysis confirmed that there was no significant breakdown of any of the antagonists used during incubation (data not shown).

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DISCUSSION

Understanding the regulation of hepatocyte function by extracellular nucleotides is complicated by the presence of multiple P2Y receptor subtypes which are activated by a range of native agonists. Here we have examined regulation of rat hepatocytes by the partially-selective synthetic ADP derivative, 2-MeSADP which acts at $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors. In a previous report, this nucleotide was shown to increase $[Ca^{2+}]_c$ (Dixon, 2000) and here we demonstrate that at similar concentrations it activates glycogen phosphorylase. However, we also report an apparently paradoxical attenuation of glucagon-stimulated cyclic AMP levels by 2-MeSADP, and have investigated this inhibitory effect to determine whether the same receptor mediates the conflicting influences on glycogen phosphorylase activity.

2-MeSADP is an agonist at the rat $P2Y_1$ receptor (Hechler et al., 1998) which is expressed by hepatocytes (Dixon et al., 2000). The potency of 2-MeSADP to stimulate glycogen phosphorylase (EC_{50} 121 nM) is consistent with this response being mediated by $P2Y_1$ receptors, and this is confirmed by inhibition by the competitive and selective $P2Y_1$ antagonist, MRS 2179. We report a parallel shift to the right of the concentration-response curve for 2-MeSADP in the presence of this drug. The apparent pA_2 value of 6.82 compares well with the reported pA_2 of 6.99 for MRS 2179 at the turkey erythrocyte $P2Y_1$ receptor (Boyer et al., 1998).

Characteristically there was a small secondary rise in glycogen phosphorylase activity at the highest concentrations of 2-MeSADP tested. This may reflect the accumulation of the products of 2-MeSADP degradation. As described in the results section, incubation of freshly-isolated cells with 2-MeSADP led to accumulation of 2-methylthio-substituted derivatives of adenosine and AMP. These compounds could activate adenosine A_{2B} receptors present on rat hepatocytes which are coupled to increased cyclic AMP levels (Yasuda et al., 2003). This could conceivably stimulate glycogen phosphorylase further through the classical pathway via protein kinase A activation.

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In single rat hepatocytes, 2-MeSADP evokes oscillations in $[Ca^{2+}]_c$ at concentrations as low as 100 nM, consistent with a role in the $P2Y_1$-mediated activation of glycogen phosphorylase as described above. Furthermore, these oscillations were blocked by the $P2Y_1$ receptor antagonist A3P5P (Dixon, 2000). The $P2Y_1$ receptor however, does not appear to mediate the inhibition of glucagon-stimulated cyclic AMP accumulation by 2-MeSADP observed here, as this effect was not reversed by $P2Y_1$ receptor antagonists. Interestingly, both A3P5P and MRS 2179 had significant inhibitory effects on adenylyl cyclase in hepatocytes which could potentially complicate the interpretation of experiments using these compounds. However, the maximal ability of these two drugs to directly attenuate cyclic AMP levels was substantially smaller than the inhibitory response to 2-MeSADP. It would therefore not completely mask an antagonist effect if 2-MeSADP was acting at $P2Y_1$ receptors. We conclude therefore that inhibition of adenylyl cyclase by 2-MeSADP is mediated by a receptor distinct from the $P2Y_1$ receptor which is responsible for increased $[Ca^{2+}]_c$ and activation of glycogen phosphorylase.

To determine which receptors may be expressed in rat hepatocytes, we have extended our previously reported RT-PCR analysis (Dixon et al., 2000) to include recently-identified members of the P2Y family. Consistent with the previous study (Dixon et al., 2000) we have shown that transcripts for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ are expressed in freshly-isolated hepatocytes, but in addition we report the expression of transcripts for $P2Y_{12}$ and $P2Y_{13}$ receptors. This is particularly significant as these receptors are negatively coupled to adenylyl cyclase through G_i (Communi et al., 2001; Zhang et al., 2002), and 2-MeSADP has been identified as an agonist at the rat $P2Y_{12}$ receptor (Hollopeter et al., 2001) and the mouse and human $P2Y_{13}$ receptors (Communi et al., 2001; Zhang et al., 2002). (The rat $P2Y_{13}$ receptor has yet to be cloned and its agonist profile is therefore unknown.)

RT-PCR results demonstrated that following culture for 48 hours, transcripts for the G_i-coupled P2Y receptors were either barely detectable ($P2Y_{12}$), or absent ($P2Y_{13}$). Experiments

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conducted in parallel with RT-PCR, revealed a concomitant large reduction in the ability of 2-MeSADP to inhibit glucagon-stimulated cyclic AMP levels. Culturing cells also led to an apparent increase in the effect of UTP, although this is likely to reflect the difference in stability of UTP under the two conditions. Significantly though the observation that UTP remains effective at inhibiting the cyclic AMP levels is consistent with the view that UTP and 2MeSADP act at separate P2Y receptor subtypes, and that the reduced response to 2-MeSADP reflects changes at the level of the receptor rather than an altered transduction mechanism.

These results suggested that the inhibitory response to 2-MeSADP may be mediated by $P2Y_{12}$ or $P2Y_{13}$ receptors. We therefore investigated the effect of AR-C67085, an antagonist with high potency (IC_{50} = 0.6 nM; Humphries et al., 1995b) at the rat $P2Y_{12}$ receptor, and low potency at the human $P2Y_{13}$ receptor (IC_{50} = 0.63 μM; Marteau et al., 2003). AR-C67085, at concentrations up to 300 μM, did not antagonize the 2-MeSADP-evoked inhibition of glucagon-stimulated cyclic AMP levels in rat hepatocytes, arguing against the involvement of $P2Y_{12}$ receptors. Assuming the pharmacology of the rat $P2Y_{13}$ receptor reflects that of the human homologue, the lack of antagonism by AR-C67085 also argues against $P2Y_{13}$ receptor involvement. The accumulation during incubations of 2MeSAMP, a known $P2Y_{12}$ and $P2Y_{13}$ antagonist (Hollopeter et al., 2001; Marteau et al., 2003), is unlikely to affect these conclusions since any antagonism would not be effective at the onset of the incubation. Furthermore, studies with adenosine 5'-O-(2-thiodiphosphate), a stable but low potency agonist of both $P2Y_{12}$ and $P2Y_{13}$ receptors (Marteau et al., 2003; Zhang et al., 2001), also showed no evidence of either of these receptors (unpublished data).

In comparison to the high potency of 2-MeSADP to evoke $[Ca^{2+}]_c$ oscillations in single rat hepatocytes (Dixon, 2000), or to activate glycogen phosphorylase as demonstrated here, the threshold concentration of 2-MeSADP required to elicit an inhibitory effect on cyclic AMP levels was high. It is however consistent with a previous report by Edgecombe et al. (1999) in which the

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inhibitory effects of ATP and Ap_4A in freshly-prepared rat hepatocytes were apparent only at concentrations greater than 100 μM. We have demonstrated by HPLC that following a 3 minute incubation of hepatocytes with 300 μM 2-MeSADP only 56% was recovered. It may therefore be assumed that in the absence of any degradation the threshold concentration of 2-MeSADP for inhibition of glucagon-stimulated cyclic AMP levels will be lower than that reported here. However, it remains apparent that the inhibition of cyclic AMP accumulation in response to P2Y receptor agonists will only be effective at concentrations which are high compared to those required to stimulate glycogen phosphorylase. The functional consequences may be to act to restrain the effects of the highest concentrations of extracellular nucleotides.

In conclusion we have demonstrated that in freshly-isolated rat hepatocytes 2-MeSADP both stimulates glycogen phosphorylase and inhibits adenylyl cyclase , and that these effects are the result of stimulation of distinct subtypes of P2Y receptor. The results of antagonist studies show that the activation of glycogen phosphorylase is mediated by the $P2Y_1$ receptor responsible for the previously reported elevation of $[Ca^{2+}]_c$ (Dixon, 2000), but that the inhibition of adenylyl cyclase is not mediated by $P2Y_1$ receptors, or any other of the characterized receptors for which 2-MeSADP is a known agonist. It is therefore possible that 2-MeSADP acts at a hitherto uncloned receptor. It has recently been recognised that the P2Y receptor family is structurally diverse, the most recently-identified receptor, $P2Y_{14}$, being a receptor for UDP-glucose (Abbracchio et al., 2003). Through phylogenetic analysis a number of orphan receptors have been identified which closely correlate with the P2Y family of G-protein-coupled receptors (Fredriksson et al., 2003), one of which may correspond to that on rat hepatocytes mediating the effect of 2-MeSADP. However, it remains a possibility that the rat $P2Y_{13}$ receptor displays an altered pharmacology with respect to antagonism by AR-C67085 compared with the human $P2Y_{13}$ receptor, and that this receptor sub-type mediates the inhibition of glucagon-stimulated cyclic AMP levels in rat

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hepatocytes. A further possibility is that receptor dimerization of known P2Y receptors results in a previously uncharacterised physiological response.

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Footnotes:

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Figure Legends

Figure1: Stimulation of glycogen phosphorylase by 2-MeSADP in freshly isolated hepatocytes, in the absence (■) and presence (○) of MRS 2179. Cells in suspension were stimulated for 2 min with the increasing concentrations of 2-MeSADP. Where indicated cells were pre-incubated with MRS 2179 (100 μM) for 10 min. Results are from a representative experiment, with stimulations performed in triplicate. Data pooled from 3 separate experiments are reported in the text.

Figure 2: Effect of 2-MeSADP and UTP on glucagon-stimulated cyclic AMP accumulation in freshly isolated and cultured hepatocytes. Cyclic AMP accumulation was measured in rat hepatocytes which were (a) freshly-isolated or (b) cultured for 48 hours and stimulated for 3 min with 10 nM glucagon and 300 μM 2-MeSADP or 300 μM UTP. Cells were derived from the same preparation. Results are from a representative experiment, with stimulations performed in triplicate. Data pooled from 3 separate experiments are reported in the text.

Figure 3: RT-PCR analysis of the expression of P2Y receptor transcripts in isolated and cultured hepatocytes. RNA extracts were subjected to RT-PCR with P2Y receptor-specific primers and primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For each primer set, lanes 1 and 3 correspond to amplifications from freshly-isolated and cultured hepatocytes respectively. Lanes 2 and 4 are the corresponding controls where reverse transcriptase was omitted. The unmarked lanes contained molecular size markers (100 bp). The figure is representative of three separate experiments.

Figure 4: Effect of the $P2Y_1$ antagonist, MRS 2179 on attenuation of glucagon-stimulated cyclic AMP levels in response to 2-MeSADP. Cyclic AMP accumulation was measured in freshly-isolated rat hepatocytes stimulated for 3 min with 10 nM glucagon and (a) 100 μM or (b) 300 μM 2-MeSADP, together with increasing concentrations of MRS 2179. Results are

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from a single representative experiment, with stimulations performed in triplicate. Data pooled from 3 separate experiments are reported in the text.

Figure 5: Effect of the $P2Y_{12}$ antagonist, AR-C67085 on attenuation of glucagon-stimulated cyclic AMP levels in response to 2-MeSADP. Cyclic AMP accumulation was measured in freshly-isolated rat hepatocytes stimulated for 3 min with 10 nM glucagon and (a) 100 μM or (b) 300 μM 2-MeSADP, together with increasing concentrations of AR-C67085. Results are from a single representative experiment, with stimulations performed in triplicate. Data pooled from 3 separate experiments are reported in the text.

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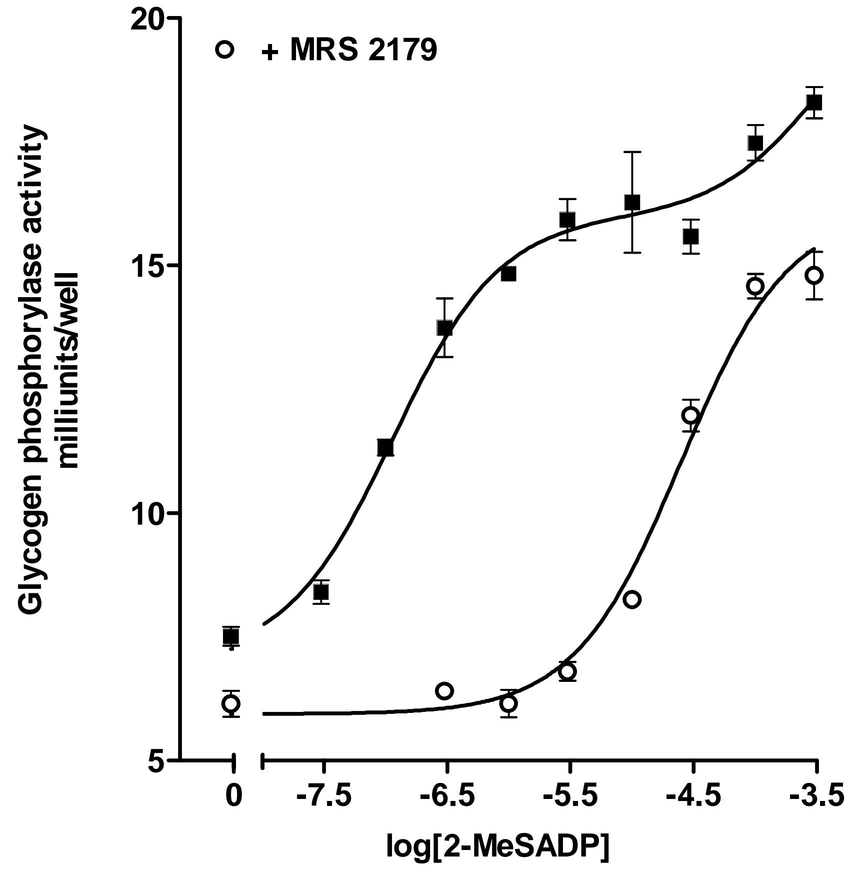
JPET #6774 Fig 1



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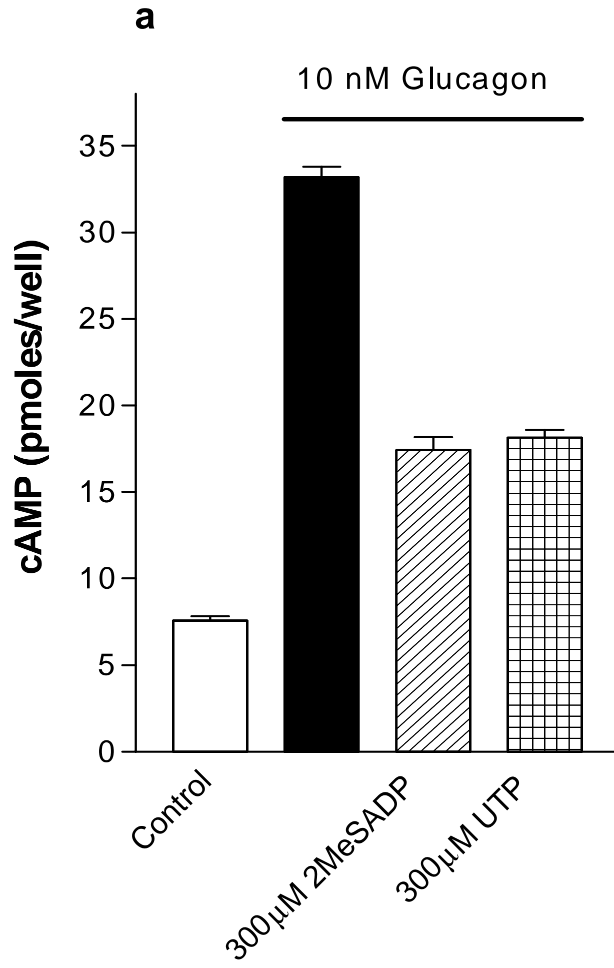

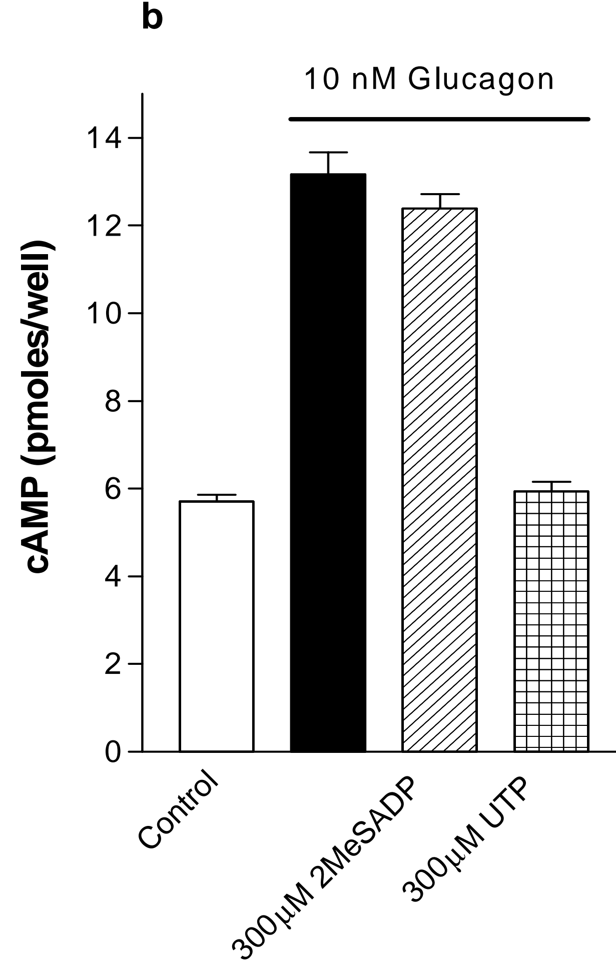

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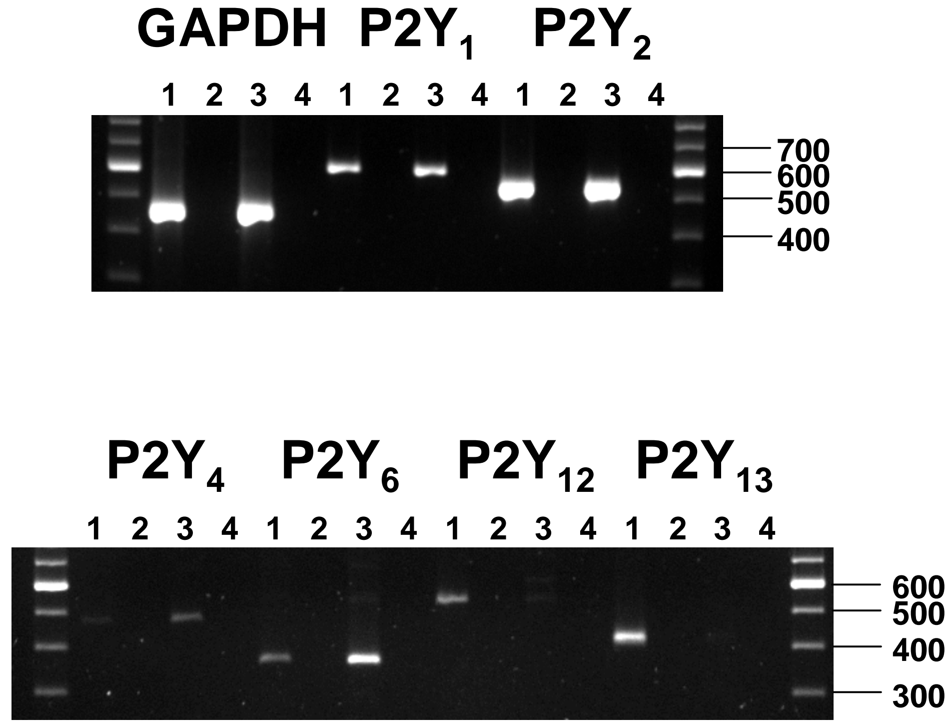
JPET 6774 Fig 3



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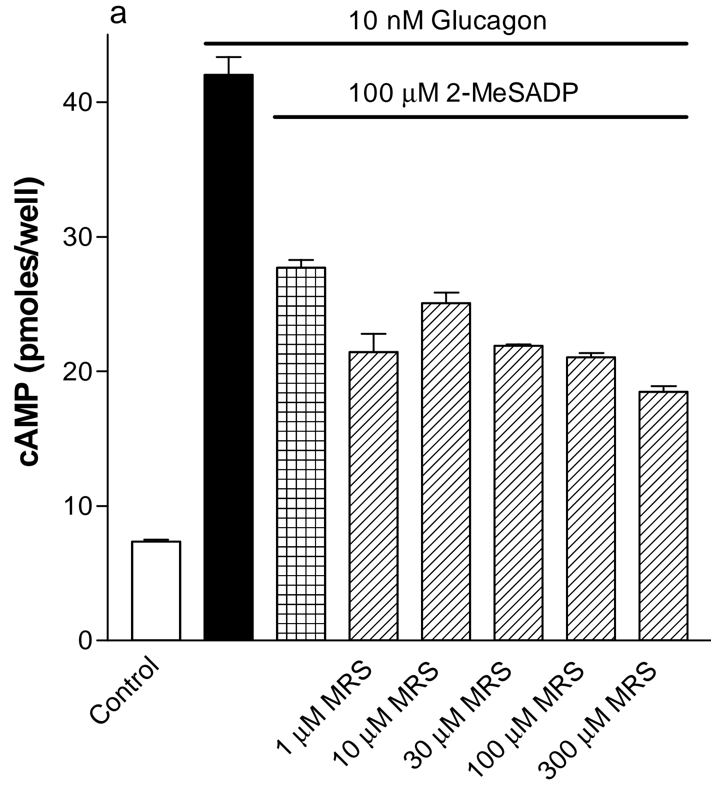
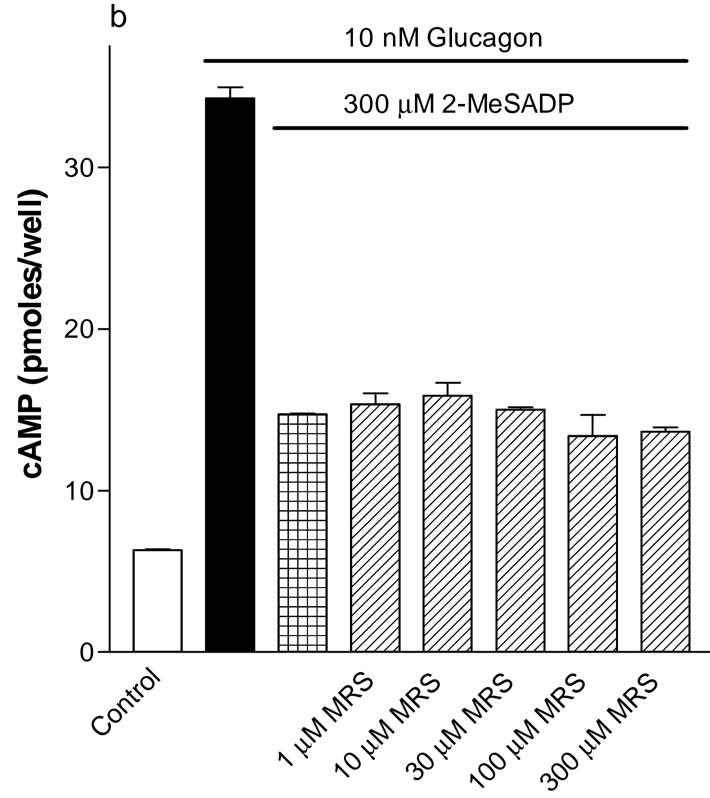
JPET 6774 Fig 4





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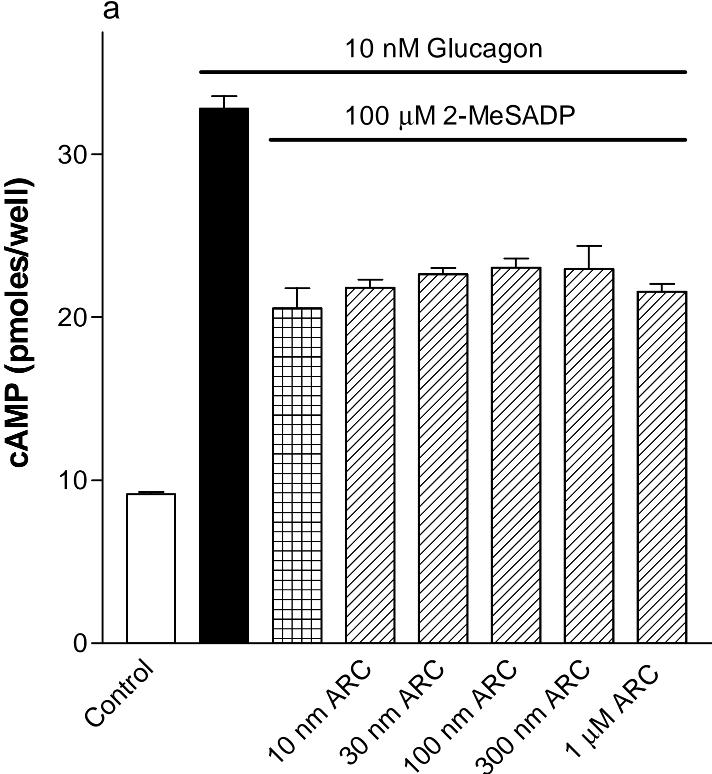
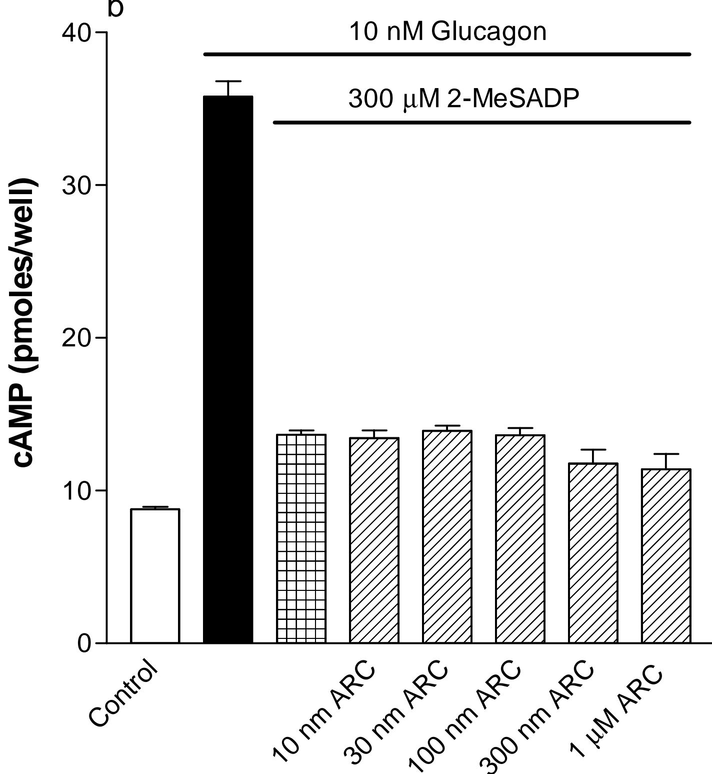
JPET 6774 Fig 5





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