Regulation of Human Hepatocytes by P2Y Receptors: Control of Glycogen Phosphorylase, Ca$^{2+}$, and Mitogen-Activated Protein Kinases

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ABSTRACT

In the rat both short-term liver function, such as glycogen metabolism, and long-term events such as proliferation after partial hepatectomy, are in part controlled by release of nucleotides such as ATP acting on hepatocyte P2Y$_1$ and P2Y$_2$ receptors (members of a family of P2Y receptors for extracellular nucleotides such as ATP and UTP). Here, we have studied P2Y$_1$ receptor regulation of signaling pathways involved in glycogen phosphorylase activation and proliferation of primary human hepatocytes. Stimulation of cultured hepatocytes with either ATP and UTP, but not UDP or 2-methylthio ADP, led to concentration-dependent increases in cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_c$; EC$_{50}$ for ATP = 3.3 μM; for UTP = 2.3 μM) and [H]$^3$H]inositol (poly)phosphates (EC$_{50}$ for ATP = 9.4 μM, for UTP = 15.4 μM). ATP and UTP also stimulated glycogen phosphorylase in human hepatocytes, each with a threshold for activation of less than 1 μM. Application of 2-methylthio ADP up to 100 μM was ineffective. Phosphorylation of both extracellular signal-related kinase and c-Jun N-terminal kinase was stimulated by ATP and UTP, but not by 2-methylthio ADP or UDP, either alone or when costimulated with epidermal growth factor. In conclusion, in human hepatocytes P2Y receptors control both glycogen metabolism and proliferation-associated responses such as increased [Ca$^{2+}$]$_c$ and mitogen-activated protein kinase cascades. Regulation seems to be primarily through P2Y$_2$ receptors. In contrast with previous studies on rat hepatocytes, there is an absence of responses mediated by P2Y$_1$ receptors.

Hepatocytes are regulated by a diverse range of agonists acting at cell surface receptors. One key, highly regulated function of hepatocytes is the supply of glucose into the bloodstream, released from glycogen stores in the liver and controlled by the enzyme glycogen phosphorylase. Early work on rat hepatocytes implicated P2Y$_2$ receptors in the regulation of glycogen metabolism. These are a family of G protein-coupled receptors for extracellular nucleotides such as ATP, UTP, ADP, UDP, and UDP-glucose (Boarder and Hourani, 1998; Abbracchio et al., 2003). Stimulation of P2Y$_2$ receptors leads to increases in cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_c$), and hence activation of phosphorylase kinase, which in turn phosphorylates and activates glycogen phosphorylase. In rat hepatocytes, increases in [Ca$^{2+}$]$_c$ in response to extracellular nucleotides are mediated by both P2Y$_1$ and P2Y$_2$ receptors (Okajima et al., 1987; Dixon et al., 1990, 2000, 2003; Keppens, 1993). It has been explicitly demonstrated that activation of P2Y$_1$ receptors in rat hepatocytes substantially stimulates glycogen phosphorylase (Dixon et al., 2004). Cyclic AMP also plays a role in control of rat hepatic glycogen phosphorylase, but here nucleotides exert an inhibitory influence.

Another highly regulated function in hepatocytes is cell proliferation. Ca$^{2+}$ signaling plays a role in the control of cell division for many cell types (Berridge et al., 2000), although there is no direct evidence for this in hepatocytes. In healthy adult liver, hepatocytes do not enter the cell cycle. However, after partial hepatectomy hepatocytes divide to restore liver mass. This regulated entry into the cell cycle involves a complex convergence of signals (Fausto, 2004). In vivo, rat hepatocytes proliferate in response to established mitogens such as epidermal growth factor (EGF). The three main mitogen-activated protein kinase (MAPK) cascades [extracellular signal-related kinase (ERK), c-Jun NH$_2$-terminal kinase (JNK), and p38 MAPK] are implicated in hepatocyte proliferation, apoptosis, and survival (Ulrich et al., 1998; Melien et

ABBREVIATIONS: [Ca$^{2+}$]$_c$, cytosolic Ca$^{2+}$ concentration; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; JNK, c-Jun NH$_2$-terminal kinase; [H]$^3$H]InsP$_x$, [H]$^3$H]inositol (poly)phosphates; HPLC, high-performance liquid chromatography; CPK, creatine phosphokinase; AR-C67085MX, (2-propylthio-β, -dichloromethylene-β-ATP).
JNK activation in particular is implicated in the mitogenic response to ATP (Thevananther et al., 2004) and is also known to be a very early event after partial hepatectomy in rat (Westwick et al., 1995), when hepatocytes swell, inducing release of ATP (Feranchak et al., 2000) and activating JNK and ERK pathways (Kim et al., 2000). This suggests that stimulation of P2Y receptors and consequent JNK activation may contribute to cell cycle progression in the rat liver after partial hepatectomy.

There is little information on regulation of primary human hepatocytes by P2Y receptors. It has been shown that ATP activates glycogen phosphorylase (Keppens et al., 1993), and ATP and UTP elicit indistinguishable \([\text{Ca}^{2+}]\) responses (Schofl et al., 1999). Furthermore, in these human cells EGF stimulates DNA synthesis (Kaminski et al., 1986), and EGF and insulin activate ERK1,2 (Ulrich et al., 1998). Here, we provide a characterization of nucleotide-stimulated responses related to both glycogen phosphorylase and proliferation in primary human hepatocytes.

### Materials and Methods

#### Cell Preparation and Culture.

Human hepatocytes were prepared by the United Kingdom Human Tissue Bank (De Montfort University, Leicester, UK) from liver obtained at resection surgery, with ethical committee approval from both donor institution and De Montfort University, and with informed consent of donor patients. The tissue was perfused with a \(\text{Ca}^{2+}\)-free buffer and then collagenase, followed by sequential filtration to yield a suspension of single cells. Hepatocytes were then separated from nonparenchymal cells by centrifugation, resulting in a preparation of greater than 95% cells. Hepatocytes were resuspended in William’s Medium supplemented with 10% fetal calf serum, 50 IU/ml penicillin, 50 \(\mu\)g/ml streptomycin, and 50 \(\mu\)g/ml gentamicin, and plated at a density of 1 \(\times\) \(10^5\) cells/well in collagen-coated 24-well plates for inositol phosphate studies. 6 \(\times\) \(10^5\) cells/well in collagen-coated six-well plates for the glycogen phosphorylase assay and Western blots. For \([\text{Ca}^{2+}]\), studies, cells were plated at 1 \(\times\) \(10^5\) cells/well in 12-well plates containing 12-mm-diameter collagen-coated coverslips.

Rat hepatocytes were isolated from fed, male Wistar strain rats (150–250 g) by collagenase perfusion as described previously (Dixon et al., 1995). The cells were harvested and cultured as described above for human cells.

#### Total \([3H]\)InsP\(_3\) (Poly)phosphates.

Cells were cultured for 24 h and then labeled for 48 h at 37°C in 5% \(\text{CO}_2\) with myo-[\(3H\)]inositol (0.037 MBq/ml; 0.5 ml/well) in serum-free medium M199.

Twenty-minute stimulations, in the presence of 10 mM LiCl, were made without change of medium. The reaction was stopped with trichloroacetic acid, the solution was neutralized with freon-octylamine extraction and addition of NaHCO\(_3\), and total \([3H]\)InsP\(_3\) was extracted on small Dowex-1 (Cl\(^-\)) columns. The dose-response curves for \([3H]\)InsP\(_3\) did not saturate, true EC\(_{50}\) values could not be reported. Instead, we report apparent EC\(_{50}\) values calculated as the concentration of agonist that generates 50% of the maximal response achieved.

### High-Performance Liquid Chromatography of Nucleotides: Assessment of Breakdown.

High-performance liquid chromatography (HPLC) was used to assess nucleotide purity and to monitor nucleotide breakdown by cultured cells. An Alltech SAX column was used with a Waters HPLC system (gradient between 0.05 M KH\(_2\)PO\(_4\) and 1 M KH\(_2\)PO\(_4\)), with data collection and peak integration by Millenium 32 software. Stock (10 mM) solutions of ATP and UTP were incubated with a regenerating system comprising 10 mM creatine phosphate and 20 U/ml creatine phosphokinase (CPK). UDP (10 mM) was treated with 80 U/ml hexokinase and 110 mM glucose. These enzymes are therefore also present in the incubations with cells, maintaining levels of added nucleotides. To assess nucleotide breakdown during the \([3H]\)InsP\(_3\) assays, incubations were conducted in parallel but in the absence of radioactivity, and samples were analyzed by HPLC.

### Cyclic AMP Measurement.

Cells were cultured in 24-well plates (24–48 h in culture) with 300 \(\mu\)M 3-isobutyl-1-methylxanthine for 10 min before agonist addition. Cells were stimulated by nucleotides (300 \(\mu\)M, except where otherwise indicated) and/or glucagon (10 nM) for 3 min. Incubations were terminated by addition of trichloroacetic acid. Cyclic AMP was extracted with a 1:1 mixture of tri-n-octylamine and 1,1,2-trichloro-trifluoroethane and assayed using the protein binding assay of Brown et al. (1971).

### Glycogen Phosphorylase Assay.

Cells were cultured for between 12 and 18 h in William’s medium E as described above. This was replaced with HEPES-buffered medium (116 mM NaCl, 5.6 mM KCl, 0.8 mM MgSO\(_4\), 1.1 mM KH\(_2\)PO\(_4\), 4.8 mM NaHCO\(_3\), 11 mM glucose, 1.8 mM CaCl\(_2\), and 20 mM HEPES, pH 7.4) 1 h before stimulation. Cells were stimulated with agonists for 2 min, and incubations were terminated by replacing medium with 150 \(\mu\)l of extraction buffer (100 mM NaF, 20 mM EDTA, 0.5% (v/v) glycerol, and 50 mM glycyl glycine, pH 7.4). Cells were homogenized, and extracts were frozen immediately in liquid nitrogen and then subjected to three cycles of freeze thawing before centrifugation at 10,000g for 1 min at 4°C. A 100-\(\mu\)l portion of supernatant was incubated with 20 \(\mu\)l of assay buffer [600 mM glucose-1-phosphate, 1.8 M NaF, 6 mM caffeine, 12% (v/v) glycerol, and 29.6 KBq/ml \([^{14}C]\)glucose-1-phosphate, pH 6.1] for 40 min at 30°C. The reaction was terminated by spotting onto filter papers, which were washed in ethanol for 1.5 h, briefly soaked in acetone, and dried before being counted for radioactivity.

### Western Blots.

Cells were cultured overnight and then maintained serum free for 24 h before stimulation with agonists for the times indicated, followed by addition of liquid N\(_2\). Extracts were prepared for Western blots in ice-cold lysis buffer (20 mM Tris-HCl, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 1 mM \(\beta\)-mercaptoethanol, 0.5% Triton X-100 (\(\nu/\nu\)), 20 \(\mu\)g/ml aprotinin, and 5 \(\mu\)g/ml leupeptin, pH 7.6). The sonicated lysate was spun at 10,000g for 10 min at 4°C. Samples were equalized for protein and run on 10% polyacrylamide gels. Western blots, prepared using polyclonal antibodies specific for the phosphorylated forms of ERK, JNK, and p38 MAPK, were developed with enhanced chemiluminescence reagent.

### Data Analysis.

Data processing, including curve fitting, determination of EC\(_{50}\) values, and statistical analysis, was undertaken with GraphPad Prism (GraphPad Software Inc., San Diego, CA). Data are expressed in the figures and text as mean ± S.E. Materials. AR-C67085MX was a kind gift form AstraZeneca (Charnwood, Loughborough, UK). Cell culture medium was from Invitrogen (Paisley, UK). Myo-[\(2^\text{H}\)]inositol was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK).
UK). Collagenase was from Roche Diagnostics (Lewes, UK), EGF from AL-ImmunoTools (Friesoythe, Germany), and fura 2-acetoxyethyl ester from Calbiochem (Merck Biosciences, Nottingham, UK). Antisera specific for the phosphorylated (activated) forms of phospho-ERK, phospho-JNK, and p38 MAPK were from Promega (Madison, WI). CPK, phosphorecatine, nucleotides, and all other chemicals were from Sigma Chemical Co. (Poole, Dorset, UK).

**Results**

**Increases in \([\text{Ca}^{2+}]_c\), Stimulated by Nucleotides in Human Hepatocytes.** Fura 2-loaded hepatocytes were stimulated with nucleotides, and the ratio of fluorescence emitted at 540 nm with sequential excitation at 340 and 380 nm was plotted as an index of \([\text{Ca}^{2+}]_c\). Figure 1, A–D, show typical responses elicited by 30-s stimulations with agonists at 100 \(\mu\)M. The \([\text{Ca}^{2+}]_c\) response to both ATP and UTP (Fig. 1, A and B) consisted of a rapid rise, followed by a slow decline to baseline. All cells responded to both ATP and UTP with evidence of oscillations in \([\text{Ca}^{2+}]_c\), in some cells. Exposure to 100 \(\mu\)M UDP (with hexokinase) gave variable results, with a modest rise in \([\text{Ca}^{2+}]_c\), in some cells, yet no response in others (Fig. 1C). In stark contrast to the effects of ATP and UTP, 100 \(\mu\)M 2-methylthio ADP did not elicit a rise in \([\text{Ca}^{2+}]_c\) (Fig. 1D). The concentration-response curves for the four agonists generated from data pooled across three separate experiments are shown in Fig. 1E. The potencies of both ATP and UTP are within the range expected for activation of P2Y receptors: \(EC_{50}\) for ATP was 3.33 \(\mu\)M (\(-\log EC_{50} = 5.48 \pm 1.76\)) and \(EC_{50}\) for UTP was 2.31 \(\mu\)M (\(-\log EC_{50} = 5.64 \pm 0.01\)). Contrasting with this, UDP and 2-methylthio ADP failed to generate a reliable response at less than 300 \(\mu\)M. We also examined the effects of the P2Y11 agonist AR-C67085MX and the P2X agonist \(\alpha,\beta\)-methylene ATP. Neither evoked a response at concentrations up to 100 \(\mu\)M (data not shown).

In a previous study on rat hepatocytes, we showed that the expression of mRNA transcripts for selected P2Y receptors, and certain responses to 2-methylthio ADP, decline over 48 h in culture (Dixon et al., 2004). To determine whether a similar effect accounts for the lack of response to 2-methylthio ADP in human hepatocytes, we investigated cells cultured for only 2 h. Results shown in Fig. 2 indicate that although these cells exhibited a strong response to ATP and UTP (Fig. 2, A and B), they did not respond to 2-methylthio ADP or UDP (Fig. 2, C and D).

**Increases in \([\text{Ca}^{2+}]_c\), Stimulated by Nucleotides in Rat Hepatocytes.** The lack of response to 2-methylthio ADP was surprising because this agonist is known to be effective at rat hepatocytes (Dixon, 2000). To determine whether 2-methylthio ADP is also an agonist under the conditions used here, rat hepatocytes were cultured on coverslips and stimulated in parallel with human hepatocytes. As expected rat hepatocytes responded to ATP and UTP with concentration-dependent increases in \([\text{Ca}^{2+}]_c\) (not shown). A maximal response was achieved at 100 \(\mu\)M; examples from individual cells are illustrated in Fig. 3, A and B. In keeping with previous reports (Keppens, 1993; Dixon et al., 2000), the responses from individual cells to ATP and UTP were indistinguishable, with a rapid increase followed by a plateau phase. These similarities indicate a common receptor. Hepatocytes derived from the same cell preparation failed to respond to 100 \(\mu\)M UDP (Fig. 3C). However, in common with human hepatocytes, the effect of UDP was variable, with a clear response recorded from some cells. In contrast to human hepatocytes, 2-methylthio ADP was a reliable and potent agonist. Typical examples of the \([\text{Ca}^{2+}]_c\) responses to 0.3 \(\mu\)M 2-methylthio ADP are shown in Fig. 3D. The response to 2-methylthio ADP differed to that evoked by ATP and UTP with a more rapid falling phase and the absence of a plateau. These differences are consistent with the previous assertion that 2-methylthio ADP acts at a distinct receptor from ATP and UTP (Dixon et al., 2000).

**Accumulation of \([^{3}\text{H}]\text{InsP}_4\).** Stimulation of \([^{3}\text{H}]\text{inositol-labeled human hepatocytes with ATP and UTP (both treated with CPK) led to a concentration-dependent accumulation of }^{[3}\text{H}]\text{InsP}_4\). Figure 4 shows concentration-response curves for ATP and UTP and illustrates the similarity between the responses to these two nucleotides. Because the responses failed to reach a plateau, apparent \(EC_{50}\) values were calculated (see Materials and Methods) and yielded a value for ATP of 9.43 \(\mu\)M (-\(\log EC_{50} = 5.03 \pm 0.15\), and for UTP of 15.38 \(\mu\)M (-\(\log EC_{50} = 4.81 \pm 0.18\)). Figure 4 also shows that there were no detectable responses to 2-methylthio ADP or UDP, up to maximal concentrations of 300 \(\mu\)M.

**Nucleotide Breakdown.** The extent of nucleotide breakdown under conditions for the \([^{3}\text{H}]\text{InsP}_4\) assay was assessed by HPLC. After addition of 300 \(\mu\)M agonist, and incubation for 20 min, the percentage of nucleotide remaining was ATP, 101 \(+9\%\); UTP, 75 \(+16\%\); UDP, 96 \(+27\%\); and 2-methylthio ADP, 69 \(+11\%\).

**Accumulation of Cyclic AMP.** To determine whether nucleotides affect cyclic AMP synthesis, cultured human hepatocytes were stimulated with nucleotides alone or in the presence of glucagon, which stimulates cyclic AMP synthesis. Nucleotides alone (300 \(\mu\)M) had no effect on cyclic AMP accumulation in any of the experiments undertaken (data not shown). As expected glucagon (10 nM) reliably increased cyclic AMP levels in human hepatocytes, and this stimulation was inhibited by ATP, UTP, and UDP. There was considerable variability between cell preparations in the basal cyclic AMP concentration, the degree of stimulation achieved by glucagon, and the extent of the inhibition in response to nucleotides. However, nucleotides typically reduced the glucagon stimulation by between 30 and 70\%, as illustrated in Fig. 5. In contrast 2-methylthio ADP had no significant effect on glucagon-stimulated cyclic AMP levels in any experiment. Further concentration-response curves (0.01–100 \(\mu\)M) showed no consistent effects of this nucleotide, although in some experiments there was a small yet unreliable reduction in glucagon-stimulated cyclic AMP levels in response to submicromolar levels of 2-methylthio-ADP (data not shown).

**Activation of Glycogen Phosphorylase in Cultured Human Hepatocytes.** Figure 6A shows the activation of glycogen phosphorylase in a typical experiment in response to a number of nucleotide agonists (100 \(\mu\)M). As illustrated, 2-methylthio ADP was found to be ineffective (-fold increase over basal 1.14 \(+0.05\), pooled across three separate experiments). Stimulation with 100 \(\mu\)M UDP yielded a small activation of glycogen phosphorylase in cultured human hepatocytes, although this was not consistent (1.35 \(+0.69\), over three separate experiments). Application of UTP or ATP, however, produced a potent activation and concentration-response curves to these two agonists were constructed (Fig.
6B, data pooled across three separate experiments, each in triplicate, and expressed as a percentage of the maximal response). The mean EC₅₀ for ATP was 5.45 μM (–log EC₅₀ = 5.22 ± 0.06), compared with 3.02 μM (–log EC₅₀ = 5.51 ± 0.09) for UTP. The two curves were not significantly different (P > 0.05 by two-way analysis of variance).

Fig. 1. Increases in [Ca²⁺], induced by extracellular nucleotides in human hepatocytes cultured for 24 h. Responses of individual fura 2-loaded cells to stimulation with ATP (A), UTP (B), UDP (C), and 2-methylthio ADP (D), each at 100 μM, are shown. ATP and UTP included the CPK regenerating system, and UDP included the hexokinase regenerating system, as described under Materials and Methods. Agonist application was for 30 s in each case, as shown by the bar. Pooled concentration-response curves are shown (E) from several experiments (mean ± S.E. 2-methylthio-ADP).
Fig. 2. Increases in \([\text{Ca}^{2+}]_c\) induced by extracellular nucleotides in human hepatocytes cultured for 2 h. Responses of individual fura 2-loaded cells to stimulation with ATP (A), UTP (B), UDP (C), and 2-methylthio ADP (D), each at 100 
\([\mu\text{M}],\) are shown. ATP and UTP included the CPK regenerating system, and UDP included the hexokinase regenerating system, as described under Materials and Methods. Agonist application was for 30 s in each case, as shown by the bar.

Fig. 3. Increases in rat hepatocyte \([\text{Ca}^{2+}]_c\) induced by extracellular nucleotides. Responses of individual fura 2-loaded cells to stimulation with ATP (A), UTP (B), UDP (C), and 2-methylthio ADP (D), are shown. ATP and UTP included the CPK regenerating system, and UDP included the hexokinase regenerating system, as described under Materials and Methods. Agonist application was for 30 s in each case, as shown by the bar.
Activation of MAPK Pathways. No detectable increase in p38 MAPK phosphorylation was observed in response to ATP, UTP, 2-methylthio ADP, or UDP at 100 μM, with various durations of stimulation (data not shown). However, as shown in Fig. 7, stimulation with ATP or UTP enhanced phosphorylation of both p42 and p44 ERK. This was apparent by 2 min, and maximal at 5 to 10 min, with a decline by 30 min. The p42 form showed the largest degree of phosphorylation. However, in response to 2-methylthio ADP and UDP, a reliable increase in ERK phosphorylation was not detected.
Similarly, ATP and UTP produced a time-dependent increase in phosphorylation of both p46 and p54 JNK, but in this case the response was typically slower, peaking at 10 to 20 min (Fig. 7). JNK phosphorylation was not detected in response to UDP or 2-methylthio ADP, as illustrated in Fig. 8B, which shows responses to nucleotides in the presence and absence of EGF. The phosphorylation of ERK and JNK in response to 20 ng/ml EGF was similar to that seen with ATP or UTP (Fig. 8). Costimulating for 5 min with either ATP or UTP and EGF did not increase the degree of ERK phosphorylation with either agonist alone. However, with the weaker response at 20 min, the ERK phosphorylation seemed additive when ATP or UTP was combined with EGF. The JNK response was greater at 20 min than at 5 min, as predicted from Fig. 7, and again the response at 20 min was approximately additive when ATP or UTP was combined with EGF. No responses to UDP or 2-methylthio ADP were seen under any of the conditions tested.

**Discussion**

In this study, we have investigated short-term responses to extracellular nucleotides in human hepatocytes. We report that P2Y receptors regulate each of the cellular mechanisms investigated and that the underlying pharmacology of the human cells is fundamentally different from that of rat hepatocytes.

There are currently eight cloned subtypes of human P2Y receptors: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14 (Boarder and Hourani, 1998; Barnard and Simon, 2001; Abbracchio et al., 2003), each with a characteristic agonist profile. The four agonists used in the present study (ATP, UTP, UDP, and 2-methylthio ADP) provide a degree of discrimination between these receptors. 2-methylthio ADP is a full and potent agonist at P2Y1, P2Y12, and P2Y13 receptors (Zhang et al., 2002; Marteau et al., 2003). ATP and UTP act identically as agonists at human P2Y2 receptors. ATP is also an agonist at P2Y13 receptors. The nucleotide derivative AR-C67085MX is a selective agonist at P2Y11 receptors (Communi et al., 1999) and is therefore a useful tool to test for the involvement of this receptor subtype. UTP acts at P2Y4 receptors: ATP is not an effective agonist at human P2Y4 receptors, differing in this respect from rat P2Y4 receptors, at which both ATP and UTP are full agonists (Kennedy et al., 2000). UDP is a full agonist at P2Y6 receptors (Communi et al., 1996). Selective antagonists are available only for P2Y1, P2Y12, and P2Y13 receptors.

In their native environment, the majority of P2Y receptors are coupled to activation of phospholipase C, formation of inositol 1,4,5-trisphosphate, and the consequent elevation of \([Ca^{2+}]_c\), which in hepatocytes will result in activation of glycogen phosphorylase. The increases in \([Ca^{2+}]_c\) we report
here in response to ATP and UTP show a rapid rise to peak, followed by a slow decline, typical of responses mediated by G protein-coupled receptors. Furthermore the concentration-response curves generate EC_{50} values in the low micromolar range, typical of responses to native P2Y receptors. The similarity of responses to ATP and UTP suggests involvement of P2Y_{13} receptors. ATP is also an agonist at P2Y_{13} receptors. However, the lack of response to AR-C67085MX indicates that activation of the P2Y_{11} receptor does not contribute to the ATP-evoked increase in [Ca^{2+}]_c in human hepatocytes. UTP also acts as an agonist at the human P2Y_{4} receptor. Since ATP is not an agonist at this receptor subtype (Kennedy et al., 2000), the ATP response would have to be at an as yet unidentified receptor. Action of ATP and UTP at a common P2Y_{2} receptor is by far the most likely explanation.

2-Methylthio ADP did not induce an increase in [Ca^{2+}]_c in human hepatocytes. This was surprising because previous experiments have demonstrated that this agonist potently elevated [Ca^{2+}]_c in single aequorin-injected rat hepatocytes (Dixon, 2000). In this previous study, rat hepatocytes were not cultured but suspended in an agarose matrix. We therefore cultured rat cells under the conditions used for human hepatocytes, to investigate the possibility that this difference is the result of the conditions used, rather than a genuine species difference. In these experiments cultured rat hepatocytes loaded with fura-2 generated a robust and highly potent response to 2-methylthio ADP.

These results clearly establish that the Ca^{2+} response of rat hepatocytes to 2-methylthio ADP is maintained over several days in culture. However, we have previously found that the inhibition of glucagon-stimulated cyclic AMP accumulation declined in culture, as did expression of mRNA transcripts of P2Y_{12} and P2Y_{13} (Dixon et al., 2004). This raised the possibility that human cells may lose the Ca^{2+} response to 2-methylthio ADP after 2 days in culture. We therefore investigated the responses of cells cultured for only 2 h, the minimum possible for [Ca^{2+}]_c measurements, and show that even at this time 2-methylthio ADP is not effective. We therefore conclude that the lack of response to this agonist was not due to prolonged time in culture.

This work reveals that human hepatocytes do not express the P2Y_{2} receptors coupled to increased [Ca^{2+}]_c that are present in rat cells and that ATP and UTP stimulate increases in [Ca^{2+}]_c by acting primarily at P2Y_{2} receptors. The lack of response to 2-methylthio ADP additionally shows that P2Y_{12} and P2Y_{13} receptors are not responsible for regulating [Ca^{2+}]_c in the human cells. The failure to detect a potent and reliable response to UDP indicates that P2Y_{6} receptors are not consistently present. These conclusions are strengthened by the results of experiments measuring phospholipase C activation ([3H]InsP_{4} accumulation), inhibition of glucagon-stimulated cyclic AMP levels, and glycogen phosphorylase activation; in each case, ATP and UTP gave essentially the same pattern of stimulation, UDP did not give a reliable or full response, and 2-methylthio ADP was ineffective. This indicates that the P2Y_{2} receptors in human hepatocytes are coupled to [Ca^{2+}]_c by activation of phospholipase C and generation of inositol 1,4,5-trisphosphate and that this elevated [Ca^{2+}]_c leads to stimulation of glycogen phosphorylase. The physiological consequences of P2Y_{2} receptor stimulation would therefore be expected to include release of glucose into the bloodstream. The role of P2Y_{2} receptors is therefore similar in rat and human. However, in the rat, glycogen phosphorylase is also potently regulated by activation of P2Y_{1} receptors (Dixon et al., 2004). This contrasts with the results presented here, which indicate that glycogen phosphorylase is not regulated by P2Y_{1} receptors in human hepatocytes.

The recent study by Thevananther et al. (2004) has established a role for P2Y receptors in the regulation of proliferation-related responses in rat hepatocytes, showing that the stable nucleotide ATP-S stimulates responses associated with cell cycle progression, and that this requires activation of signaling through the JNK pathway, one of the MAPK cascades. Our own unpublished work shows that ATP and 2-methylthio ADP are able to stimulate MAPK pathways in cultured rat hepatocytes, which combined with other studies indicates that both P2Y_{1} and P2Y_{2} receptors are likely to play a significant role in the regulation of hepatocyte proliferation in the rat. The MAPK cascades provide the classical pathways coupling cell surface growth factor receptors to the cell cycle. We provide evidence here that two MAPK pathways, ERK and JNK, are stimulated by ATP and UDP acting on human hepatocytes. This evidence is based upon the detection, with phospho-specific antibodies, of dual Thr/Tyr phosphorylation required for the activation of these kinases. In parallel experiments p38 phosphorylation was not detected. Furthermore, we did not see any evidence of stimulation of the MAPK pathways studied by either 2-methylthio ADP or UDP. ATP- and UTP-stimulated phosphorylation of ERK was maximal at about 5 min, whereas the stimulation of JNK phosphorylation peaked at 10 to 20 min. Synergism between P2Y receptor stimulation and EGF in the activation of proliferation-related events has been reported in rat hepatocytes (Thevananther et al., 2004). Here, we show that EGF stimulates both ERK and JNK (but not p38 MAPK) phosphorylation in human hepatocytes. Under certain conditions of stimulation the effect of EGF and nucleotides together can be seen to be greater than the effect of either agonist alone. These results indicate that the ERK and JNK pathways are regulated by P2Y_{2} receptors (but not by P2Y_{1}, P2Y_{4}, P2Y_{6}, P2Y_{12}, or P2Y_{13} receptors) in human hepatocytes. The JNK pathway has been widely associated with the promotion of apoptosis, and yet various reports have shown growth factor stimulation of both JNK and ERK (Chen et al., 1996; Higashita et al., 1997). There is also evidence for a role for JNK in the receptor-stimulated proliferation of smooth muscle cells (Kyaw et al., 2001; Schmitz et al., 2001), as well as rat hepatocytes (Thevananther et al., 2004).

It can be concluded therefore that human hepatocyte P2Y_{2} receptors regulate short-term responses, such as increased [Ca^{2+}]_c and glycogen phosphorylase activation, which need to be controlled on a minute-by-minute basis in a healthy liver. In addition, ATP and UTP stimulate responses associated with long-term changes in hepatocyte function, such as proliferation, which may be required in a damaged liver. These findings are likely to be of physiological significance: nucleotides reach micromolar levels in the liver (Chari et al., 1996), and native ATP released from hepatocytes has an autocrine role (Chari et al., 1996; Schlosser et al., 1996, Wang et al., 1996). The differences between rat and human hepatocyte P2Y receptors predict a difference in regulation by native purine nucleotides: rat hepatocytes will be stimulated by both ADP (acting at P2Y_{1} receptors) and ATP (acting at
P2Y<sub>2</sub> receptors), whereas in human liver ADP will not be effective. Recently, in vivo studies in rat have demonstrated that activation of P2Y<sub>2</sub> receptors leads to stimulation of MAPK pathways after partial hepatectomy (Thevananther et al., 2004). This highlights the importance of the observations reported here in understanding regulatory mechanisms in the regenerating liver.

Acknowledgments

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