Purinergic receptors in human placenta: evidence for functionally active P2X4, P2X7, P2Y2, and P2Y6

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Roberts, V. H. J., S. L. Greenwood, A. C. Elliott, C. P. Sibley, and L. H. Waters. Purinergic receptors in human placenta: evidence for functionally active P2X4, P2X7, P2Y2, and P2Y6. Am J Physiol Regul Integr Comp Physiol 290: R1374–R1386, 2006.—Appropriate regulation of ion transport by the human placental syncytiotrophoblast is important for fetal growth throughout pregnancy. In nonplacental tissues, ion transport can be modulated by extracellular nucleotides that raise intracellular calcium ([Ca2+]i) via activation of purinergic receptors. We tested the hypothesis that purinergic receptors are expressed by human placental cytotrophoblast cells and that their activation by extracellular nucleotides modulates ion (K+) efflux and [Ca2+]i. P2X2/P2X7 receptor agonists 5-bromouridine 5'-triphosphate (5-BrUTP), ADP, ATP, 2'-3'-O-(4-benzoyl-benzoyl)adenosine 5'-triphosphate (BzATP), and UTP stimulated 86Rb (K+ tracer) efflux from cultured cytotrophoblast cells at early (mononuclear) or later (multinucleate syncytiotrophoblast-like) stages of differentiation, with ATP and UTP particularly potent. 2-Methylthioadenosine 5'-triphosphate (2-MeS-ATP), and UDP elevated 86Rb efflux only from multinucleated cells. All agonists caused a significant peak and plateau increase in [Ca2+]i, although the magnitude of responses was variable. The effect of BzATP, ATP, UTP, and UDP in multinucleated cells was unaffected, and that of ATP partially inhibited, by removal of extracellular Ca2+; potassium; cytotrophoblast

THE SYNCTIOTROPHOBLAST is the solute-transporting epithelium of the human placenta and facilitates maternal-fetal nutrient exchange. The syncytiotrophoblast continuously develops and differentiates over the course of pregnancy, and throughout this process, solute transport must be appropriately regulated to support the growing fetus. Because the human placenta is not innervated (42), autocrine, paracrine, or endocrine modulation of syncytiotrophoblast transport function assumes particular importance.

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In nonplacental tissues, the extracellular nucleotides ATP and UTP, and their metabolites ADP and UDP, regulate a variety of cell functions, including electrolyte transport, volume homeostasis, cytokine secretion, and apoptosis (3, 6, 8, 16), by activating purinergic receptors and raising intracellular Ca2+ concentration ([Ca2+]i). Purinergic receptors can be divided into two groups based on their molecular structure and signal transduction mechanisms (7). The P2X receptor subgroup comprises seven members (P2X1–P2X7) that are ligand-gated Ca2+-permeable ion channels that open after the binding of an extracellular nucleotide (5). The P2Y receptors are G protein coupled, and agonist binding activates phosphoinositide breakdown to mobilize Ca2+ from intracellular stores (7). Fifteen members of the P2Y family have been reported (P2Y1–P2Y15), although some are only related through weak homology and several (P2Y5, P2Y7, P2Y9, P2Y10) do not function as receptors that raise [Ca2+]i (44).

In nonplacental epithelia, cellular nucleotides are released constitutively (16, 62), but secretion can be enhanced by stimuli such as mechanical or shear stress, cell swelling, hypoxia, inflammation, or platelet activation (21, 31). In the kidney and intestine, secretion of cellular nucleotides raises their concentration in extracellular fluid sufficient to activate purinergic receptors and modulate electrolyte transport (34, 55). Ectonucleotidases on the apical plasma membrane of these tissues hydrolyze the secreted nucleotides and control their local concentrations (64). Although nucleotide secretion has not been measured from the human placenta, it is possible that nucleotides are released constitutively or that secretion is stimulated by mechanical/shear stress generated by the flow of maternal blood that makes direct contact with the syncytiotrophoblast microvillous membrane; consistent with this, ectonucleotidases have been localized to this plasma membrane (9, 37). Furthermore, nucleotide secretion may be enhanced in diseases of pregnancy, such as preeclampsia or intrauterine growth restriction, which are associated with fetal hypoxia, abnormal placental oxygenation, and platelet aggregation (20, 38). It is therefore plausible that extracellular nucleotides may modulate syncytiotrophoblast solute transport physiology and pathophysiology.

Under primary culture conditions, mononuclear cytotrophoblast cells isolated from human term placenta mimic the behavior of cytotrophoblast cells in vivo by fusing and differentiating to form multinucleate syncytiotrophoblast-like cells. Accordingly, these cells have been widely used as an in vitro
model of the syncytiotrophoblast and a system in which to examine trophoblast cell differentiation from the mono- to multinucleate phenotype (11, 22). Our group (12) previously demonstrated that extracellular ATP elevates \([\text{Ca}^{2+}]_i\) and promotes K\(^+\) efflux from multinucleate cytotrophoblast cells, although the receptors involved were not identified. In the present study, we tested the hypothesis that multiple purinergic receptor subtypes are expressed by mono- and multinucleate cytotrophoblast cells and that their activation by extracellular nucleotides modulates ion (K\(^+\)) efflux and \([\text{Ca}^{2+}]_i\). We studied the effect of a panel of purinergic receptor agonists on K\(^+\) \((^{86}\text{Rb})\) efflux rate and \([\text{Ca}^{2+}]_i\), and determined the expression of mRNA and protein for P2X and P2Y receptors by cytotrophoblast cells at early (mononucleated) and later ( multinucleated) stages of culture.

**METHODS**

**Materials.** 2-Methylthioadenosine 5\’-diphosphate (2-MeS-ADP), 2-methylthioadenosine 5\’-triphosphate (2-MeS-ATP), 5-bromouridine 5\’-triphosphate (5-BrUTP), \(\alpha\)-methyladenosine 5\’-triphosphate (\(\alpha\)-mATP), ADP, ATP, 2,3\’-O-(4-benzoyl-benzoyl)adenosine 5\’-triphosphate (BzATP), UDP, and UTP were purchased from Sigma-Aldrich (Poole, UK), and fura-2 AM was purchased from Molecular Probes Europe (Leiden, The Netherlands). All other reagents were analytical grade and purchased from standard suppliers.

**Cell isolation and culture.** All studies were performed in accordance with the Declaration of Helsinki, and the local ethics committee approved all procedures. Cytotrophoblast cells were isolated using the method described previously in detail (11, 22), modified from the procedure originally described by Kliman et al. (29). Briefly, placentas were collected within 30 min of delivery, and villous tissue was dissected, roughly minced, and washed with 0.9% saline. The tissue was digested in trypsin (Invitrogen, Paisley, UK) with DNase (Sigma-Aldrich) in a HEPES-buffered saline solution, and isolated cells were separated by centrifugation through a discontinuous Percoll gradient. Cytotrophoblast cells were plated at a density of ~4.5 \times 10^6 cells per 35-mm culture dish for efflux experiments or ~1.5 \times 10^6 cells per flamed 16-mm diameter glass coverslip for microfluorimetry and maintained in a humidified incubator at 37\(^\circ\)C in 5% CO\(_2\)-95% air for up to 3 days of culture. Investigators in our laboratory (11, 22) have previously shown that during this time, cytotrophoblast cells undergo both morphological and biochemical differentiation to form multinucleated syncytiotrophoblast-like cells. To examine whether the activity and expression of purinergic receptors altered with differentiation, we studied cytotrophoblast cells at two time points in culture: after 18 h, when the majority of cells are mononuclear, and after 66 h, when the majority of cells are multinucleate and resemble the syncytiotrophoblast in situ (11, 22).

**\(^{86}\text{Rb} \text{ efflux.}** \(^{86}\text{Rb} \text{ efflux was assessed from cytotrophoblast cells under control conditions and in response to a panel of P2Y and P2X receptor agonists (2-Mes-ADP, 2-Mes-ATP, 5-BrUTP, \(\alpha\)-MeATP, ADP, ATP, BzATP, UDP, and UTP) using the methods previously described (12). Briefly, cytotrophoblast cells were loaded for 2 h with a 3 \mu M/\text{ml stock solution of }^{86}\text{Rb} \text{ in control Tyrode’s solution (135 mM NaCl, 5 mM KCl, 1 mM MgCl}_2, 1.8 mM CaCl}_2, 5.6 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 with 10 M NaOH) at room temperature. After loading, cells were washed for 3 min with 50 ml of control Tyrode’s solution to remove extracellular isotopes. Fresh solution (1 ml) was then applied and collected at 1-min intervals over a 10-min experimental period. Tyrode’s solution containing agonist was applied each minute from 5 min onward. At the end of the experiment, the cells were lysed for 2 h in 0.3 M NaOH. Cell lysates and efflux samples were counted for \(^{86}\text{Rb} \text{ activity with a Packard gamma counter.**

**Experimental groups.** In an initial screening study, each agonist was applied at a single concentration (100 \mu M) to cells isolated from the same placenta, to eliminate variability between placentas [our group previously found that 100 \mu M ATP produced maximal \(^{86}\text{Rb} \text{ efflux from cytotrophoblast cells (12).** From these data, the rate constant of the fall in cellular isothe with time in controls was compared with that in the presence of each agonist on cells from the same isolate. A concentration-response study was subsequently performed for each agonist over the concentration range 1 \mu M-1 mM.

**Contamination of commercial ADP and UDP with ATP and UTP, respectively, was assessed by treating the stock solutions with hexokinase. Treatment with hexokinase is a standard approach that has been used previously to decontaminate commercial preparations of ADP (36) and UDP (39). A 10-mM stock solution of ADP or UDP was prepared in hexokinase buffer (150 mM NaCl, 10 mM HEPES, 2.5 mM KCl, 1 mM MgCl\(_2\), 2.5 mM CaCl\(_2\), and 22 mM glucose, adjusted to pH 8.0 with NaOH) with 3 U/ml hexokinase (Roche Diagnostics) and was incubated for 1 h at 37\(^\circ\)C before use (stock diluted 1:20). This procedure reduces the triphosphate contaminants to insignificant concentrations (36).

**Calculation of \(^{86}\text{Rb} \text{ efflux results.** The \(^{86}\text{Rb} \text{ efflux rate constants were calculated from plots of ln [Rb]}_t/(Rb]}_0\) as a function of time, where Rb}(t = 0) are the counts associated with the cells at the start of the time course and Rb}(t) are the counts remaining in the cells at time t. The time course of \(^{86}\text{Rb} \text{ efflux (%/min) was calculated as }^{86}\text{Rb} \text{ in 1-min efflux sample cell/}^{86}\text{Rb at start of 1-min collection) } \times 100.

**Microfluorimetry.** Cells were loaded with 4 \mu M fura-2 AM (prepared from a 1 mM stock solution in DMSO) in control Tyrode’s solution containing 1% BSA for 30 min at 37\(^\circ\)C. After loading, the coverslip was transferred to a perfusion chamber (Warner Instruments, Hamden, CT). The perfusion chamber was mounted on a Nikon Diaphot inverted microscope, and cells were superfused with control Tyrode’s solution at ~2 ml/min. A field of view was selected, and six cell-containing regions were defined for analysis of \([\text{Ca}^{2+}]_i\). Mononuclear cytotrophoblast cells were chosen for experiments at 18 h of culture, and multinucleated areas (3 or more nuclei per cell) were selected at 66 h.

After a 2-min equilibration period, the cells were stimulated by the addition of ATP, BzATP, 2-MeS-ATP, \(\alpha\)-MeATP, ADP, UDP, UTP, or 5-BrUTP for 2 min. The concentration of agonist used was determined (empirically) from the \(^{86}\text{Rb} \text{ efflux experiments as that inducing the maximum stimulation of }^{86}\text{Rb} \text{ efflux. For those agonists that are thought to act at P2Y receptors (ADP, UDP, UTP, 5-BrUTP) along with ATP and BzATP (P2Y and P2X agonists), the effect of the agonist on }[[\text{Ca}^{2+}]_i\text{ also was examined in Ca}^{2+}\text{-free conditions; an increase in }[[\text{Ca}^{2+}]_i\text{, in response to agonists in the absence of extracellular }\text{Ca}^{2+}\text{ can be attributed to mobilization of }\text{Ca}^{2+}\text{ from intracellular stores following the activation of P2Y receptors. Experiments were repeated in Ca}^{2+}\text{-free Tyrode’s solution (as described in }^{86}\text{Rb efflux but with no added CaCl}_2\text{ and containing 0.5 mM EGTA), which was present for 6 min before agonist application, for the duration of agonist exposure (2 min), and for a further minute after agonist removal. All experiments were carried out at room temperature.}

**The fluorescence imaging system was controlled by a personal computer running the Kinetic Imaging (Nottingham, UK) AQM Advance software package, driving an Optoscan monochromator (Cairn Research, Faversham, UK) and a Hamamatsu cooled charged-coupled device camera (model OrcaER) to allow image acquisition at 340 and 380 nm. Ratio images were constructed off-line, and six cells in each image were selected for analysis. The 340- to 380-nm fluorescence ratio was calculated as a measure of \([\text{Ca}^{2+}]_i\). Two time points were selected for analysis of the agonist-induced effect on \([\text{Ca}^{2+}]_i\); the time of maximal increase in ratio (peak) and 2 min after the peak, which has been referred to as the plateau. The control ratio was measured in the 2 min preceding agonist application. In a few experiments with \(\alpha\)-MeATP and 2-MeS-ATP, a well-defined peak
increase in \([\text{Ca}^{2+}]\) was not observed in mononucleated cells. In these experiments, the ratio was measured 10 s after addition of the agonist, because this corresponded to the average time that a peak increase was detected in responsive cells.

**RT-PCR.** Gene-specific primers for the purinergic receptor subtypes P2X1, P2X2, P2X3, P2X4, P2X5, P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 were designed for use in RT-PCR. Primers of 20 base pairs (bp) in length were selected to amplify partial cDNA from each sequence (Table 1). Total RNA, prepared from cytotrophoblast cells at 18 and 66 h of culture (\(n = 3\) placental preparations), was reverse transcribed (Invitrogen) for use in PCR by using a Thermocycler (Perkin Elmer). For PCR amplification, samples were denatured at 95°C for 2.5 min, followed by a protocol consisting of 35 cycles of 1 min at 95°C, 1 min of annealing at \(T_m\), where \(T_m\) is the primer-specific annealing temperature (Table 1), and 1 min of extension at 72°C. A further 5 min at 72°C ensured cDNA extension. PCR products were separated on a 1.2% agarose gel containing ethidium bromide for visualization over UV light. Identity of PCR products was determined by restriction enzyme digest and sequencing. Negative controls omitted cDNA; positive control amplifications used DNA derived from tissues known to express the receptor subtype of interest.

**Western blotting.** Protein was extracted from cytotrophoblast cells (\(n = 3\) placental preparations) after centrifugation of cells treated in PBS (Alomone Laboratories). For preabsorption, P2X4, P2X7, P2Y2, and P2Y6 antibodies were incubated with 1 μg of peptide per 1 μg of antibody for 1 h at room temperature before incubation with the membrane as described above.

After primary antibody incubation, the membranes were incubated with either horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma-Aldrich; purinergic receptors and negative control) diluted 1:8,000 in PBS containing 5% milk protein or horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (Sigma-Aldrich; \(\beta\)-actin) diluted 1:1,000 in TBS-0.1% Tween containing 5% milk protein. Signals were detected with an enhanced chemiluminescence detection system (ECL; Amersham Biosciences) and detected on photosensitive film (Hyperfilm-ECL; Amersham Biosciences).

**Data analysis and statistics.** The data are expressed as means ± SE, with \(n\) representing the number of placentas from which cells were isolated. Least-squares linear regression was used to analyze the decrease in \(^{86}\text{Rb}\) with time \([\ln [\text{Rb}]_t(0)/[\text{Rb}]_t(t=0)]\); see Calculation of \(^{86}\text{Rb}\) efflux results), and the rate constants for \(^{86}\text{Rb}\) efflux were taken as the negative slope of the regression line fitted over the experimental period (6–10 min). To analyze the agonist concentration-response data, we combined the %\(^{86}\text{Rb}\) efflux/2 min and plotted the data as percentage increase above control. Statistical analyses of the differences in \(^{86}\text{Rb}\) efflux in control conditions and in response to agonists were performed on the raw data by using repeated-measures ANOVA with a Dunnett’s multicomparison post hoc test.

Microfluorimetry data are presented as the number of individual cell observations per coverslip (\(n = 6\)) from each placental cytotrophoblast isolation (\(n = 6\)). To test whether each agonist significantly raised \([\text{Ca}^{2+}]\) above control (baseline), the 340/380 fluorescence ratios for control vs. peak and control vs. plateau were analyzed using a Wilcoxon matched-pairs test. To compare the responses to agonists in the presence and absence of extracellular \(\text{Ca}^{2+}\), we expressed the data as the percentage change in ratio above control. This avoids the complications associated with calibrating fura-2 measurements to estimate \([\text{Ca}^{2+}]\), concentration, and percentage changes have been used previously to express \([\text{Ca}^{2+}]\), in cytotrophoblast cells after ATP stimulation (12).

The effects of 2-MeS-ATP and \(\alpha\beta\)-meATP on \([\text{Ca}^{2+}]\), were assessed using a paired Student’s t-test to compare the percentage change in 340/380 ratio at the peak and plateau response. For the remaining agonists, peak and plateau were compared in both \(\text{Ca}^{2+}\) -containing and \(\text{Ca}^{2+}\)-free conditions by using repeated-measures ANOVA and a Bonferroni post hoc test comparing all groups. The changes in \([\text{Ca}^{2+}]\), in response to agonists in cytotrophoblast cells at 18 and 66 h of culture were compared using an unpaired Student’s t-test. \(P\) values <0.05 were considered significant.

Table 1. Primer sequences used in PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Tm, °C</th>
<th>Length, bp</th>
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<tbody>
<tr>
<td>P2X1 5′:</td>
<td>5′-ctacagggagccagccagc-3′</td>
<td>54</td>
</tr>
<tr>
<td>3′:</td>
<td>5′-gtagagcagctcagctcag-3′</td>
<td>59</td>
</tr>
<tr>
<td>P2X2 5′:</td>
<td>5′-ctacagggagccagccagc-3′</td>
<td>55</td>
</tr>
<tr>
<td>3′:</td>
<td>5′-catcagggagccagccagc-3′</td>
<td>57</td>
</tr>
<tr>
<td>P2X3 5′:</td>
<td>5′-ctacagggagccagccagc-3′</td>
<td>56</td>
</tr>
<tr>
<td>3′:</td>
<td>5′-ctacagggagccagccagc-3′</td>
<td>56</td>
</tr>
<tr>
<td>P2Y1 5′:</td>
<td>5′-gtacacaaaggaagcagc-3′</td>
<td>54</td>
</tr>
<tr>
<td>3′:</td>
<td>5′-cgacaaaggaagcagc-3′</td>
<td>60</td>
</tr>
<tr>
<td>P2Y2 5′:</td>
<td>5′-gatgtagagcagccagccagc-3′</td>
<td>56</td>
</tr>
<tr>
<td>3′:</td>
<td>5′-gatgtagagcagccagccagc-3′</td>
<td>58</td>
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<tr>
<td>P2Y3 5′:</td>
<td>5′-acccgagccagcagccagc-3′</td>
<td>53</td>
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<tr>
<td>3′:</td>
<td>5′-acccgagccagcagccagc-3′</td>
<td>53</td>
</tr>
</tbody>
</table>

Annealing temperature \((T_m)\) and expected length (bp) of the PCR product are given for each primer pair.

**RESULTS**

**Measurement of \(^{86}\text{Rb}\) (K+ ) efflux.** The effects of ATP, ADP, BzATP, UTP, UDP, 5-BrUTP, 2-MeS-ATP, 2-MeS-ADP, and \(\alpha\beta\)-meATP at 100 μM were initially examined on cells isolated from the same placenta (\(n = 3\) placentas). The rate constants, calculated as the slopes of the least-squares linear regression lines fitted over the period of agonist application and...
corresponding control period (6–10 min), are given in Table 2. Under both control and experimental conditions, a single exponential gave a statistically significant fit, indicating that \( ^{86}\text{Rb} \) efflux from mono- and multinucleated cytotrophoblast cells in response to each agonist is predominantly through a single exit pathway or, less likely, several pathways with identical rate constants.

**Basal \(^{86}\text{Rb} \) efflux.** Basal (unstimulated) \(^{86}\text{Rb} \) efflux from cytotrophoblast cells at both 18 and 66 h of culture was stable over 3–10 min (see Figs. 1 and 4). The total basal \(^{86}\text{Rb} \) efflux from unstimulated cells over 6–10 min (i.e., the sum of %\(^{86}\text{Rb} \) efflux each min for minutes 6–10, which correspond to the experimental period in the treatment groups) was 7.4 ± 0.39% for cells at 18 h and 4.8 ± 0.25% for cells at 66 h of culture (\( P < 0.0001, \) unpaired t-test; \( n = 27 \) cell preparations).

**Purinergic agonist concentration-response profile for \(^{86}\text{Rb} \) efflux.** Figures 2 and 3 show the concentration-response relationship between purinergic receptor agonists and \(^{86}\text{Rb} \) efflux. Because the basal efflux rate was greater in mono- compared with multinucleated cells, the data are expressed as percent increase above control to facilitate comparison between the effects of the agonists at different stages of culture.

With the exception of 2-MeS-ADP and 2-MeS-ATP (Fig. 3), all the purinergic receptor agonists tested stimulated \(^{86}\text{Rb} \) efflux, and the percent increase above control was greater in multi- than in mononucleated cells (Fig. 2). ATP, ADP, BzATP, and 5-BrUTP (Fig. 2, A–C, and F) significantly raised \(^{86}\text{Rb} \) efflux above basal levels at both stages of culture, and the concentration-response profiles were similar in mono- and multinucleated cells.

UTP had a potent effect on mononucleated cytotrophoblast cells, inducing a significant increase in \(^{86}\text{Rb} \) efflux at all concentrations from 1 to 500 \( \mu \text{M} \), but in multinucleated cells, only 500 \( \mu \text{M} \) UTP elevated \(^{86}\text{Rb} \) efflux significantly above control (Fig. 2D). UDP had no significant effect on \(^{86}\text{Rb} \) efflux at early stages of culture but raised efflux in differentiated cells at 100 and 500 \( \mu \text{M} \) (Fig. 2E). Neither 2-MeS-ATP nor 2-MeS-ADP had any effect on \(^{86}\text{Rb} \) efflux (Fig. 3, A and B). Finally, \( \text{αβ}-\text{meATP} \) did not alter efflux from cells at 18 h, but a small response was induced at 500 \( \mu \text{M} \) after 66 h of culture (Fig. 3C).

**Contamination of commercial ADP and UDP sources.** Previous reports have suggested that nucleotide diphosphates, such as ADP and UDP, may be contaminated with their triphosphate counterparts and that this contamination may provide a false-positive result. Thus, to determine whether the responses induced by ADP and UDP were specific or attributable to contamination with ATP and UTP, respectively, we treated ADP and UDP stocks with hexokinase before use in \(^{86}\text{Rb} \) efflux experiments. Hexokinase breaks down any contaminating triphosphates with no effect on the diphosphate content. At early and later stages of culture, the increase in \(^{86}\text{Rb} \) efflux with 500 \( \mu \text{M} \) ADP was slightly, but significantly, reduced after hexokinase treatment (Fig. 4, A and B). For mononucleated cells, the reduction in total efflux was 22% (measured over the 4 min of agonist treatment), whereas for multinucleated cells, the reduction was 30%. The increase in \(^{86}\text{Rb} \) efflux from cytotrophoblast cells in response to 500 \( \mu \text{M} \) UDP was unaffected by hexokinase treatment (Fig. 4, C and D).

**Agonist stimulated \([\text{Ca}^{2+}]_\text{i}\).** ATP (100 \( \mu \text{M} \)), BzATP (500 \( \mu \text{M} \)), 2-MeS-ATP (500 \( \mu \text{M} \)), and \( \text{αβ}-\text{meATP} \) (1 mM), ADP (500

### Table 2. \(^{86}\text{Rb} \) efflux rate constants for 18-h and 66-h cytotrophoblast cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Slope, ( \text{min}^{-1} \times (10^{-3}) )</th>
<th>( r^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-17.01 ± 0.6</td>
<td>0.991</td>
</tr>
<tr>
<td>ATP</td>
<td>-43.89 ± 3.4</td>
<td>0.964</td>
</tr>
<tr>
<td>UTP</td>
<td>-29.28 ± 1.6</td>
<td>0.982</td>
</tr>
<tr>
<td>UDP</td>
<td>-26.92 ± 1.0</td>
<td>0.990</td>
</tr>
<tr>
<td>2-MeS-ADP</td>
<td>-20.96 ± 0.3</td>
<td>0.998</td>
</tr>
<tr>
<td>2-MeS-ATP</td>
<td>-23.95 ± 0.4</td>
<td>0.997</td>
</tr>
<tr>
<td>BzATP</td>
<td>-35.92 ± 2.4</td>
<td>0.973</td>
</tr>
<tr>
<td>5-BrUTP</td>
<td>-31.78 ± 1.5</td>
<td>0.986</td>
</tr>
<tr>
<td>ADP</td>
<td>-39.80 ± 2.6</td>
<td>0.974</td>
</tr>
<tr>
<td>66 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-12.08 ± 0.5</td>
<td>0.988</td>
</tr>
<tr>
<td>ATP</td>
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<td>0.941</td>
</tr>
<tr>
<td>UTP</td>
<td>-29.28 ± 1.6</td>
<td>0.961</td>
</tr>
<tr>
<td>UDP</td>
<td>-27.32 ± 1.7</td>
<td>0.975</td>
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<tr>
<td>2-MeS-ADP</td>
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<tr>
<td>2-MeS-ATP</td>
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</tr>
<tr>
<td>BzATP</td>
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<td>0.963</td>
</tr>
<tr>
<td>5-BrUTP</td>
<td>-30.42 ± 2.1</td>
<td>0.971</td>
</tr>
<tr>
<td>ADP</td>
<td>-25.08 ± 2.1</td>
<td>0.961</td>
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Values are means ± SE; \( n = 3 \). Slope was calculated as \( \text{ln}[\text{Rb}_{i(t)}/\text{Rb}_{i(0)}] \) where \( \text{Rb}_{i(t)} \) is the count associated with the cell at the start of the time course and \( \text{Rb}_{i(0)} \) is the count remaining at time \( t \), 2-MeS-ADP, 2-methylthioadenosine 5’-diphosphate; 2-MeS-ATP, 2-methylthioadenosine 5’-triphosphate; BzATP, 2’,3’-O-(4-benzoyl-benzoyl)adenosine 5’-triphosphate; 5-BrUTP, 5-bromouridine 5’-triphosphate.

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**Fig. 1.** Time course of \(^{86}\text{Rb} \) efflux in response to varying concentrations of ATP for mononucleated (18 h; A) and multinucleated (66 h; B) cytotrophoblast cells. Horizontal bars indicate 5-min ATP application. Dotted boxes indicate the first 2 min of agonist application that were used to compare the effects of agonists on \(^{86}\text{Rb} \) efflux from cytotrophoblast cells (see METHODS).
μM), UDP (500 μM), UTP (10 μM), and 5-BrUTP (500 μM) induced a peak increase in \([\text{Ca}^{2+}]_i\), followed by a plateau. For all agonists, the 340/380 ratio values at the peak and plateau were significantly higher than control in the presence and absence of extracellular \(\text{Ca}^{2+}\) \((P < 0.008; \text{Wilcoxon matched-pairs test; data not shown}).

Figures 5 and 6 show the percent change in \([\text{Ca}^{2+}]_i\) above control after a 2-min application of purinergic receptor agonists.

**Fig. 2.** Total \(^{86}\text{Rb}\) efflux over the initial 2-min experimental period expressed as a percentage above control from mononucleated (18 h) and multinucleated (66 h) cytotrophoblast cells in response to ATP (A), ADP (B), 2',3'-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate (BzATP; C), UTP (D), UDP (E), and 5-bromouridine 5'-triphosphate (5-BrUTP; F). Values are means ± SE, \(n = 3\) placentas. * \(P < 0.05\); ** \(P < 0.01\) vs. control (ANOVA with Dunnett’s multiple comparisons test).

**Fig. 3.** Total \(^{86}\text{Rb}\) efflux over the initial 2-min experimental period expressed as a percentage above control from mononucleated (18 h) and multinucleated (66 h) cytotrophoblast cells in response to 2-methylthioadenosine 5'-triphosphate (2-MeS-ATP; A), 2-methylthioadenosine 5'-diphosphate (2-MeS-ADP; B), and αβ-methyleneadenosine 5'-triphosphate (αβ-meATP; C). Values are means ± SE, \(n = 3\) placentas. * \(P < 0.05\) vs. control (ANOVA with Dunnett’s multiple comparisons test).
nists. All agonists induced a peak increase in [Ca$^{2+}$], that was significantly higher than the plateau in mono- and multinucleated cells in both the presence and absence of extracellular Ca$^{2+}$. The only exception was the response to BzATP in multinucleated (66 h) cells, where the initial peak increase in [Ca$^{2+}$] was maintained during the plateau phase in the presence, but not the absence, of extracellular Ca$^{2+}$ (Fig. 6B, peak +Ca vs. plateau +Ca not significant), consistent with a sustained entry of Ca$^{2+}$ through P2X receptors.

The P2X receptor agonists αβ-meATP and 2-MeS-ATP induced a significant increase in [Ca$^{2+}$], as indicated by Wilcoxon matched-pairs analysis. However, the overall response to these agonists was relatively small (Figs. 5, C and D, and 6, C and D; note the difference in scale on the y-axis), and a peak increase in [Ca$^{2+}$] was not induced by αβ-meATP and 2-MeS-ATP in all cells (see example traces, Figs. 5D and 6C).

In mononucleated cells, the increase in [Ca$^{2+}$], in response to ATP and ADP (Fig. 5, A and E) was unaffected by Ca$^{2+}$-free conditions, showing that the response was dominated by intracellular Ca$^{2+}$ release and thus implicating activation of P2Y receptors. However, both the peak and plateau rise in [Ca$^{2+}$], following BzATP, UDP, UTP, and 5-BrUTP (Fig. 5, B, F, G, and H) were significantly reduced in 0 Ca$^{2+}$, suggesting that these agonists can activate both P2X and P2Y receptors.

In contrast to mononucleated cells, the peak and plateau increases in [Ca$^{2+}$], evoked by ATP and ADP in multinucleated cells were significantly reduced in 0 Ca$^{2+}$ (Fig. 6, A and E), consistent with their stimulating both P2X and P2Y receptors (peak in 0 Ca$^{2+}$ with ADP mono- vs. multinucleated cells, $P < 0.001$). The responses to UDP and UTP (Fig. 6, F and G) also differed with stage of cell culture: UTP elicited a larger peak increase in [Ca$^{2+}$], in multinucleated cells ($P < 0.05$ vs. mononucleated), and the peak and plateau [Ca$^{2+}$] with UDP and UTP were unaffected by 0 Ca$^{2+}$ in multinucleated cells.

This suggests a predominant activation of P2Y receptors by these agonists as cytotrophoblast cells differentiate (peak in 0 Ca$^{2+}$ with UTP mono- vs. multinucleated cells, $P < 0.001$). Finally, in contrast to mononucleated cells, the peak increase in [Ca$^{2+}$] with BzATP was unaffected by 0 Ca$^{2+}$ in multinucleated cells (Fig. 6B) (activation of P2Y), and in the presence of extracellular Ca$^{2+}$, the peak and plateau [Ca$^{2+}$] did not differ, possibly indicating activation of P2X receptors. However, the changes in [Ca$^{2+}$], in response to 5-BrUTP were similar at both stages of differentiation (Figs. 5H and 6H).

In general, the increase in [Ca$^{2+}$], induced by the agonists (Figs. 5 and 6) was consistent with their ability to promote $^{86}$Rb efflux (Figs. 2 and 3). Accordingly, αβ-meATP and 2-MeS-ATP, which had weak effects on [Ca$^{2+}$], failed to stimulate $^{86}$Rb efflux, and for the other agonists, the rise in [Ca$^{2+}$] was proportionally similar to the stimulation of $^{86}$Rb efflux.

Expression of purinergic receptor mRNA. PCR products of the expected size were obtained for P2X1, P2X2, P2X4, and P2X7 and for P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 in cytotrophoblast cells at both stages of differentiation (Figs. 7 and 8). P2X3 and P2X5 mRNA could not be detected (Fig. 7). In all cases, product identification was confirmed by restriction enzyme digests and/or sequencing (data not shown).

Expression of purinergic receptor protein. The expression of P2X4, P2X7, P2Y2, P2Y4, and P2Y6 protein was assessed using Western blot analysis. Rat brain was used as a positive control for P2X7 and for P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 in cytotrophoblast cells at both stages of differentiation (Figs. 5 and 6).

Values are means ± SE, $n = 3$ placentas. *$P < 0.05$; **$P < 0.01$, ADP vs. hexokinase-treated ADP (ANOVA with Bonferroni’s post hoc test).

Fig. 4. Time course of $^{86}$Rb efflux in response to ADP and UDP after treatment with hexokinase to remove residual ATP and UTP, respectively. A: 500 μM ADP in 18-h cytotrophoblast cells. B: 500 μM ADP in 66-h cells. C: 500 μM UDP in 18-h cells. D: 500 μM UDP in 66-h cells. Horizontal bars indicate 5-min agonist application. ■ Control (basal) efflux; ▲ efflux in response to untreated agonist; and ▼ efflux in response to hexokinase-treated agonist. Values are means ± SE, $n = 3$ placentas. *$P < 0.05$; **$P < 0.01$, ADP vs. hexokinase-treated ADP (ANOVA with Bonferroni’s post hoc test).
Use of the antibody to P2X4 resulted in a strong band at the reported size for a glycosylated product of ~65 kDa in cells at both 18 and 66 h of culture \((n = 3)\). All bands disappeared after preincubation with the antigenic peptide (Fig. 9B). Two products were identified after probing with the P2X7 antibody: in rat brain, a band was detected at ~76 kDa \((n = 5)\), whereas in human brain and the cytotrophoblast cells, the product was ~145 kDa \((n = 3)\). Again, all bands disappeared after preabsorption with the P2X7-specific antigen (Fig. 9C). Use of the antibody to P2Y2 revealed a product in rat brain at a size of ~56 kDa, whereas in cytotrophoblast cells, the product was ~65 kDa (Fig. 9D). Despite the discrepancy between product sizes, all bands disappeared after preabsorption with the P2Y2-specific antigen. The specific band for P2Y2 \((65 \text{ kDa})\) was observed in four of six mononucleated, and in four of six multinucleated, cytotrophoblast cell preparations. Use of the antibody to P2Y4 revealed a band of the expected size of ~91 kDa in two positive control tissues, rat \((n = 4)\) and human brain \((n = 2)\) (Fig. 9E). P2Y4 was not detected in cytotrophoblast cells at either 18 \((n = 2)\) or 66 h of culture \((n = 2)\). Use of the antibody to P2X4 resulted in a strong band at the reported size for a glycosylated product of ~65 kDa in cells at both 18 and 66 h of culture \((n = 3)\). All bands disappeared after preincubation with the antigenic peptide (Fig. 9B). Two products were identified after probing with the P2X7 antibody: in rat brain, a band was detected at ~76 kDa \((n = 5)\), whereas in human brain and the cytotrophoblast cells, the product was ~145 kDa \((n = 3)\). Again, all bands disappeared after preabsorption with the P2X7-specific antigen (Fig. 9C). Use of the antibody to P2Y2 revealed a product in rat brain at a size of ~56 kDa, whereas in cytotrophoblast cells, the product was ~65 kDa (Fig. 9D). Despite the discrepancy between product sizes, all bands disappeared after preabsorption with the P2Y2-specific antigen. The specific band for P2Y2 \((65 \text{ kDa})\) was observed in four of six mononucleated, and in four of six multinucleated, cytotrophoblast cell preparations. Use of the antibody to P2Y4 revealed a band of the expected size of ~91 kDa in two positive control tissues, rat \((n = 4)\) and human brain \((n = 2)\) (Fig. 9E). P2Y4 was not detected in cytotrophoblast cells at either 18 \((n = 2)\) or 66 h of culture \((n = 2)\).
of the antibody to P2Y6 revealed weak bands at the expected size of ~110 kDa in the positive control tissue, rat brain, and in cytotrophoblast cell samples at both 18 (n = 3) and 66 h of culture (n = 3) (Fig. 9F). All negative control blots were clear with no bands detected (data not shown).

DISCUSSION

Although purinergic receptors have been implicated in the regulation of ion transport by the placenta in several studies (12, 26, 33, 43, 58), the present work is the first to systematically examine P2X and P2Y purinergic receptor subtype expression and functional activation in the human trophoblast. A range of agonists, with varying selectivity for P2X/P2Y receptors, elevated [Ca2+]i and induced K+ (86Rb+) efflux. The relative potency of endogenous nucleotides, in conjunction with the mRNA and protein expression of purinergic receptors, indicates the functional presence of P2X4, P2X7, P2Y2, and P2Y6 in cytotrophoblast cells.

Work in our laboratory (12) previously showed that K+ efflux from cytotrophoblast cells promoted by ATP was completely inhibited by charybdotoxin but unaffected by iberiotoxin or apamin, implicating intermediate-conductance Ca2+-activated K+ channels in the efflux pathway. In the present study, the agonists that stimulated K+ efflux also raised [Ca2+]i over the same time course, and it is probable that Ca2+-activated K+ channels mediated the raised K+ efflux from cytotrophoblast cells after activation of purinergic receptors.
Cytotrophoblast cell differentiation and purinergic receptors. The basal rate of $K^+$ efflux was greater in mono- than in multinucleated cells. This decrease in passive $K^+$ permeability/conductance is consistent with the depolarization of membrane potential difference that is associated with cytotrophoblast cell differentiation (22). Because of this difference in basal efflux, the percent increase in $K^+$ loss promoted by purinergic receptor agonists was greater in the more differentiated cells. However, the concentration dependence and time course of $K^+$ efflux in response to agonists did not differ markedly with the stage of culture. In accord with this, we did not observe any substantial differences in the expression of mRNA or protein for purinergic receptors (P2X4, P2X7, P2Y2, or P2Y6) by cytotrophoblast cells at early and later stages of culture.

In general, the increase in $[Ca^{2+}]_i$ induced by the agonists was consistent with their ability to promote $^{86}$Rb efflux. However, there were some differences in the effects of agonists on $[Ca^{2+}]_i$ between mono- and multinucleated cytotrophoblast cells. For example, UTP induced a greater rise in $[Ca^{2+}]_i$ in multi- than in mononucleated cells, whereas BzATP activated both P2Y and P2X receptors with a more marked effect on P2X in differentiated cells. ATP and ADP activation of P2Y receptors was greater in mono- than in multinucleated cells, whereas the opposite was true for UTP and UDP, where P2Y receptor activation was more readily apparent in differentiated cells. These differences might reflect a difference in the functional expression of P2X and P2Y receptor subtypes with differentiation to the multinucleate phenotype, although it also is possible that downstream signaling events are affected by differentiation. However, relatively high concentrations of agonists, some with poor selectivity, had to be used in these experiments to reveal functional receptors, and the significance of the differences observed in relation to activation of the receptor subtypes by endogenous nucleotides remains to be determined.

Evidence for P2X receptors in cytotrophoblast cells. Extracellular ATP activates all P2X subtypes but is a potent activator of P2X4 (40). In the present study, we have confirmed previous findings of a potent effect of ATP on $[Ca^{2+}]_i$ and $K^+$ efflux from cytotrophoblast cells (12), and in addition, we have demonstrated mRNA and protein expression for P2X4. The effect of ATP at lower concentrations is consistent with activation of P2X4, although other receptors may be stimulated at higher concentrations. αβ-MeATP is selective for P2X1 and P2X3 (2, 10) and is a partial agonist at P2X4 receptors at high concentrations. The lack of effect of αβ-MeATP at concentrations <500 μM suggests that P2X1 and P2X3 are not active in cytotrophoblast cells, and indeed, mRNA for P2X3 was not

Fig. 7. RT-PCR detection of P2X mRNA. RT-PCR was performed using gene-specific primers for P2X$_1$ (A), P2X$_2$ (B), P2X$_3$ (C), P2X$_4$ (D), P2X$_5$ (E), and P2X$_7$ (F). PCR product sizes are indicated. L, 100-bp ladder; −, negative control; +, positive control; 66, 66-h cytotrophoblast cells; 18, 18-h cytotrophoblast cells.

Fig. 8. RT-PCR detection of P2Y mRNA. RT-PCR was performed using gene-specific primers for P2Y$_1$ (A), P2Y$_2$ (B), P2Y$_3$ (C), P2Y$_4$ (D), P2Y$_6$ (E), and P2Y$_{11}$ (F). PCR product sizes are indicated. L, 100-bp ladder; −, negative control; +, positive control; 66, 66-h cytotrophoblast cells; 18, 18-h cytotrophoblast cells.
from cytotrophoblast cells, but only at relatively high concentrations of BzATP [e.g., 15 μM (19)], and the EC50 value for ATP is ~10-fold greater than that for BzATP at P2X7 (45). Indeed, in cytotrophoblast cells BzATP elevated [Ca2+]i, in the absence of extracellular Ca2+, consistent with activation of P2Y receptors and the subsequent release of Ca2+ from intracellular stores. However, the peak increase in [Ca2+]i induced in the presence of Ca2+ was significantly greater than the peak in Ca2+-free conditions, which suggests additional activation of P2X receptors by BzATP. These effects of BzATP are consistent with the findings of Divald et al. (17) that BzATP signals through P2X7 receptors in human placent al cytotrophoblast cells.

P2X7 products were detected at two different sizes in the Western blots, with a band in rat brain at the expected size of ~70 kDa and a band at ~145 kDa in the cytotrophoblast cell samples and human brain. Both the 70- and 145-kDa bands disappeared when the P2X7 primary antibody was preabsorbed with the specific antigen. Because the cell product was approximately twice the expected size, it is likely that the P2X7 dimer is expressed in cytotrophoblast cells. P2X7 can form multimers, and differential assembly of P2X7 subunits has been demonstrated (28), although the functional implications are unknown. However, both the P2X7 mRNA and protein expression appeared weak, although the results were not quantified precisely, suggesting that this receptor is not abundantly expressed in the plasma membrane of cytotrophoblast cells.

Evidence for P2Y1 and P2Y11. 2-MeS-ADP activates P2Y1 (30), but this agonist did not affect 86Rb efflux from cytotrophoblast cells at concentrations up to 1 mM. ADP is a potent P2Y1 agonist in nonplacental tissue (25, 61), and the large response to ADP in cytotrophoblast cells was greater than anticipated considering the published potencies and the lack of response to 2-MeS-ADP. We initially examined the possibility that the source of ADP was contaminated with ATP, using the method of degradation of triphosphate contaminants by incubation with hexokinase. Although we did not measure ATP levels in the present study, hexokinase treatment is reported to reduce such contamination to insignificant levels (36). Treatment of 500 μM ADP with hexokinase significantly reduced 86Rb efflux compared with untreated ADP, indicating that commercial sources of ADP are contaminated with ATP. Nonetheless, there remained a substantial elevation of 86Rb efflux after treatment that may be attributable to ADP activation of P2Y1. However, the possibility that ADP is acting at a different and as-yet unidentified receptor cannot be dismissed.

Obtaining functional evidence for P2Y11 is hampered by the lack of selective agonists for this receptor subtype. As mentioned previously, there is evidence that BzATP can activate both P2Y1 and P2Y11 receptors (40). Because the increase in [Ca2+]i induced by BzATP in cytotrophoblast cells had both an extracellular (Ca2+ entry) and intracellular (Ca2+ release) component, it is plausible that P2Y1 and/or P2Y11 are functionally expressed in trophoblast cells. P2Y1 mRNA has shown to be highly expressed by human placental tissue (35), and human P2Y11 was first isolated after screening of a placental cDNA library (13). We demonstrated the expression of mRNA for both P2Y1 and P2Y11 in cytotrophoblast cells, but because no commercial antibodies to these receptors were available at

detected. Our PCR data for P2X5 confirm a previous report (33) that this receptor subtype is not expressed in trophoblasts. 2-MeS-ATP is a partial agonist for P2X2 and has been reported to activate P2X2 at higher concentrations (23). In this study, we demonstrated a small, yet significant effect of 500 μM 2-MeS-ATP on [Ca2+]i, also supporting a role for P2X2, but not P2X5, in modulating trophoblast function. In the future, it would be useful to examine the effect of zinc, which is an agonist at P2X4 receptors (1) but an antagonist for P2X7 (60). BzATP was initially reported to be selective for P2X7 (45), but it is now evident that it also activates P2Y1 and P2Y11 receptors (40). BzATP raised [Ca2+]i, and stimulated K+ efflux from cytotrophoblast cells, but only at relatively high concentrations (100 μM and higher). This may reflect activation of receptors other than P2X7, because the latter is typically activated by low concentrations of BzATP [e.g., 15 μM (19)], and the EC50 value for ATP is ~10-fold greater than that for BzATP at P2X7 (45). Indeed, in cytotrophoblast cells BzATP elevated [Ca2+]i, in the absence of extracellular Ca2+, consistent with activation of P2Y receptors and the subsequent release of Ca2+ from intracellular stores. However, the peak increase in [Ca2+]i induced in the presence of Ca2+ was significantly greater than the peak in Ca2+-free conditions, which suggests additional activation of P2X receptors by BzATP. These effects of BzATP are consistent with the findings of Divald et al. (17) that BzATP signals through P2X7 receptors in human placental cytotrophoblast cells.

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the time of the study, further investigation is needed to determine whether P2Y1 or P2Y11 are functionally active in the human placenta.

Evidence for P2Y2/P2Y4/P2Y6. The extracellular uridine nucleotides UTP and UDP are selective for the uridine nucleotide-prefering receptor subtypes P2Y2, P2Y4, and P2Y6. UTP activates P2Y2 and P2Y4, whereas UDP and the synthetic UTP analog 5-BrUTP are both selective for P2Y6 (39). UTP had a potent effect on cytotrophoblast cells, stimulating $^{86}$Rb efflux maximally at 10 $\mu$M, which suggests a functional role for P2Y2 or P2Y4. Similarly, 10 $\mu$M UDP induced a significant rise in [$Ca^{2+}$], that was still evident in the absence of extracellular Ca$^{2+}$, consistent with activation of a P2Y receptor. A previous study by Petit and Belisle (43) also implicated a significant effect of UTP on [Ca$^{2+}$]i and a significant elevation of [Ca$^{2+}$]i. For example, placental trophoblast cells express Ca$^{2+}$-activated Cl$^{-}$ channels (27, 59), and Cl$^{-}$ efflux could accompany K$^{+}$ loss following purinergic receptor activation; this would also be expected to result in cell shrinkage. A role for ATP and UTP (via P2X and P2Y receptors) in modifying Cl$^{-}$ secretion has been shown in various epithelial cell types (56, 57).

In addition to constitutive release, ATP is secreted into the extracellular environment under circumstances of cell stress/damage (21, 55) leading to prolonged elevation of extracellular ATP and/or higher concentrations of this nucleotide. In brain, exposure to ATP released after ischemic damage can induce P2X7 pore formation, which promotes Ca$^{2+}$ entry and K$^{+}$ loss and lowers intracellular K$^{+}$ concentration. These events are associated with necrosis/apoptosis and enhanced cytokine release (63). In this regard, it is of interest that our laboratory (54) recently demonstrated an increase in IL-6 production by extracellular ATP from placental villous fragments in the presence of a proinflammatory stimulus. Furthermore, in preeclampsia, a disease of pregnancy associated with elevated cytokines and conditions known to stimulate nucleotide release from cells (abnormal placental blood flow, ischemia, platelet activation; Refs. 20, 38), there is altered trophoblast cell turnover and increased apoptosis (24). It is also intriguing that maxi-anion channels, candidate proteins for mediating ATP release from cardiomycocytes under ischemic or hypoxic conditions (18), are expressed in the microvillus membrane of the syncytiotrophoblast (4), and their open probability is reported to increase in preeclampsia (46).

In the absence of specific agonists, it is difficult to definitively identify functional receptors in a tissue such as the placenta with multireceptor expression. However, together, our data indicate a functional role for P2X7, P2X4, P2Y2, and P2Y6 in raising [Ca$^{2+}$]i and $^{86}$Rb efflux from cytotrophoblast cells at early and later stages of differentiation. Overall, our data suggest that nucleotide activation of P2Y6 can modulate cytotrophoblast cell ion transport physiology.

Role of purinergic receptors in syncytiotrophoblast of human placenta. This study has demonstrated effects of ATP, UTP, ADP, and UDP on cytotrophoblast cell physiology. Although the function of purinergic receptors in the syncytiotrophoblast remains to be confirmed, we have preliminary data demonstrating the expression of P2X4, P2X7, and P2Y2 protein in both first-trimester and term placental membrane fraction homogenates (unpublished observations), and P2X4 is immunolocalized to the syncytiotrophoblast of term placenta (47). However, nothing is known of the release of nucleotides from placental cells or of their concentration in placental extracellular fluid. In nonplacental epithelia, it is thought that ATP concentrations at the site of release can reach 1 mM, whereas circulating amounts are <1 $\mu$M (21). If the local extracellular ATP concentrations in placenta are similar, and the expression and/or sensitivity of purinergic receptor subtypes in the syncytiotrophoblast resembles those we identified in cytotrophoblast cells, then constitutive nucleotide release may be sufficient to activate trophoblast purinergic receptors with high affinity for ATP (P2X7/P2Y2). Constitutive release of UTP is reported to achieve an extracellular concentration of ~1 $\mu$M in nonplacental tissue (32), and we have demonstrated an effect of UTP on [Ca$^{2+}$]i and $^{86}$Rb efflux from cytotrophoblast cells at this concentration, probably mediated by P2Y2 receptors.

Although we have demonstrated increased K$^{+}$ loss from cytotrophoblast cells following the elevation of [Ca$^{2+}$], in response to purinergic receptor activation, it is unlikely that altered K$^{+}$ efflux is the only consequence of a transient increase in trophoblast [Ca$^{2+}$]. For example, placental trophoblast cells express Ca$^{2+}$-activated Cl$^{-}$ channels (27, 59), and Cl$^{-}$ efflux could accompany K$^{+}$ loss following purinergic receptor activation; this would also be expected to result in cell shrinkage. A role for ATP and UTP (via P2X and P2Y receptors) in modifying Cl$^{-}$ secretion has been shown in various epithelial cell types (56, 57).
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