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# Characterization of a $Ca^{2+}$ Response to Both UTP and ATP at Human $P2Y_{11}$ Receptors: Evidence for Agonist-Specific Signaling

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## ABSTRACT

Previous reports on heterologously-expressed human $P2Y_{11}$ receptors have indicated that ATP, but not UTP, is an agonist stimulating both phosphoinositidase C and adenylyl cyclase. Consistent with these findings, we report that in 1321N1 cells expressing human $P2Y_{11}$ receptors, UTP stimulation did not lead to accumulation of inositol(poly)phosphates under conditions in which ATP gave a robust, concentration-dependent effect. Unexpectedly, however, both UTP and ATP stimulated increases in cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$), with both nucleotides achieving similar $EC_{50}$ and maximal responses. The responses to maximally effective concentrations of ATP and UTP were not additive. The $[Ca^{2+}]_c$ increase in response to UTP was less dependent on extracellular $Ca^{2+}$ than was the response to ATP. AR-C67085 (2-propylthio-$\beta,\gamma$-difluoromethylene-D-ATP, a $P2Y_{11}$-selective agonist), adenosine 5′-*O*-(3-thiotriphosphate), and benzoyl ATP were all full agonists with potencies similar to those of ATP and UTP. In desensitization experiments, exposure to ATP resulted in loss of the UTP response; this response was more sensitive to desensitization than that of ATP. Pertussis toxin pretreatment attenuated the response to UTP but left the ATP response unaffected. The presence of 2-aminoethyl diphenylborate differentially affected the responses of ATP and UTP. No mRNA transcripts for $P2Y_2$ or $P2Y_4$ were detectable in the $P2Y_{11}$-expressing cells. We conclude that UTP is a $Ca^{2+}$-mobilizing agonist at $P2Y_{11}$ receptors and that ATP and UTP acting at the same receptor recruit distinct signaling pathways. This example of agonist-specific signaling is discussed in terms of agonist trafficking and differential signal strength.

Currently, seven members of the mammalian P2Y family of G protein-coupled receptors for nucleotides have been cloned. These display differential sensitivity to native nucleotide agonists (Communi et al., 1997, 2001; Fredholme et al., 1997; Boarder and Hourani, 1998; Hollopeter et al., 2001; Zhang et al., 2001). The human $P2Y_{11}$ receptor ($hP2Y_{11}$) is coupled to both phosphoinositidase C and adenylyl cyclase and has been described as a receptor for ATP, the most potent native nucleotide (Communi et al., 1997; 1999; van der Weyden et al., 2000; Qi et al., 2001). In these studies, ATP and analogs displayed greater potency and efficacy at the $hP2Y_{11}$ receptor than the equivalent diphosphates. Interestingly however, for the canine $P2Y_{11}$ receptor, the reverse is the case: ADP is more effective than ATP (Qi et al., 2001; Zambon et al., 2001; Torres et al., 2002). Pyrimidine nucleotides such as UTP have been reported to be ineffectual at $hP2Y_{11}$ receptors, based on their inability to stimulate adenylyl cyclase or inositol (poly)phosphate production (Communi et al., 1997; Zambon et al., 2001).

It has been assumed that transfected $P2Y_{11}$ receptors couple to increased cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) through stimulation of phosphoinositidase C, and subsequent mobilization of $Ca^{2+}$ from inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$]-sensitive stores. We studied the inositol-(poly)phosphate and $[Ca^{2+}]_c$ responses of 1321N1 cells transfected with $hP2Y_{11}$ receptors. We have found that UTP is a $Ca^{2+}$-mobilizing agonist, with a potency and maximal response similar to ATP. Unlike ATP however, UTP did not lead to increased inositol(poly)phosphate production in [$^3$H]inositol-labeled cells. The responses to the two nucleotides show other distinguishing features, indicating that they activate different pathways to $Ca^{2+}$ mobilization. These results are discussed in terms of agonist-specific signaling, where agonists acting at the same receptor recruit different signaling pathways.

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**ABBREVIATIONS:** $[Ca^{2+}]_c$, cytosolic $Ca^{2+}$; AR-C67085, 2-propylthio-$\beta,\gamma$-dichloromethylene-D-ATP; 2-APB, 2-aminoethyl diphenylborate; DMEM, Dulbecco's modified Eagle's medium; $InsP_x$, inositol polyphosphates; KHB, Krebs-HEPES buffer; RT-PCR, reverse transcriptase-polymerase chain reaction; Bp, base pairs; ANOVA, analysis of variance; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; AR-C67085, 2-propylthio-$\beta,\gamma$-difluoromethylene-D-ATP.

## Materials and Methods

$hP2Y_{11}$-transfected 1321N1 and untransfected control cells were a kind gift from Dr. T. K. Harden, (University of North Carolina, Chapel Hill, NC). AR-C67085 was donated by AstraZenica (Loughborough, UK). Fura 2 acetoxymethyl ester was purchased from Calbiochem (Nottingham, UK). Nucleotides, pertussis toxin, and 2-aminoethyl diphenylborate (2-APB) were from Sigma (, UK). Biotaq was purchased from Bioline (London, UK), Superscript II reverse transcriptase, RNase-free DNase I, oligonucleotides, and TRIzol were all obtained from Invitrogen (Glasgow, UK).

1321N1 neuroblastoma cells were routinely cultured in DMEM supplemented with fetal calf serum (10%), penicillin (50 U/ml), and streptomycin (50 $\mu$g/ml) unless otherwise indicated in the text. For the total [$^3$H]inositol (poly)phosphates ($InsP_x$) assay, cells at 80 to 90% confluence in 24-well plates were labeled for 24 h at 37°C in 5% $CO_2$ with [*myo*-2-$^3$H]inositol (0.037 MBq/ml; 1 $\mu$Ci/ml; 0.5 ml/well) in serum-free low-inositol medium M199. After 10 min of preincubation with LiCl (10 mM) at 37°C, agonists were added for 20 min. Inositol(poly)phosphates were extracted into 0.5 M trichloroacetic acid; after extraction with tri-*n*-octylamine/1,1,2-trichlorofluoroethane, inositol(poly)phosphates were purified on small Dowex-AG1 × 8 400 mesh ($Cl^-$) columns. Data are expressed as disintegrations per minute of [$^3$H]$InsP_x$ per well.

For measurements of $[Ca^{2+}]_c$, cells were grown on glass coverslips to ~40% confluence and then maintained for 24 h in serum-free DMEM. For some experiments, pertussis toxin (100 ng/ml) was included for 24 h. Cells were loaded for 60 min with 3 $\mu$M fura 2-acetoxymethyl ester in Krebs-HEPES buffer (KHB; 10 mM HEPES, pH 7.4) containing 1% bovine serum albumin and 0.025% pluronic acid F127 at room temperature in the dark. Coverslips were washed twice with KHB, placed in a 100-$\mu$l closed chamber on the microscope stage, and continuously perfused at 1 ml/min. Perfusion with agonists typically lasted 30 s, and responses were monitored with a VisiTech imaging system. (VisiTech International, Sunderland, UK). For calcium-free experiments, coverslips were perfused for 2 min with KHB-$Ca^{2+}$ (nominally $Ca^{2+}$-free buffer) before the addition of agonists. Data (maximal response above basal) from 8 to 10 cells were pooled for each experiment; the results presented are pooled across three separate experiments, except where otherwise indicated. [$^3$H]$InsP_x$ and fura-2 experiments were undertaken on the same days and on the same batches of cells.

For the cyclic AMP assay, cells in 24-well plates were maintained serum-free in DMEM (24 h). A 10-min preincubation at 37° with isobutylmethylxanthine (300 $\mu$M) was followed by 5-min stimulation at 37°C with forskolin (10 $\mu$M), ATP (300 $\mu$M), and UTP (300 $\mu$M) as indicated. The incubation was stopped with 0.5 M trichloroacetic acid, followed by extraction with tri-*n*-octylamine/1,1,2-trichlorofluoroethane. After neutralization with $NaHCO_3$, cyclic AMP levels were measured using the protein binding assay (Brown et al., 1971). Data, pooled across three experiments each performed in triplicate, are presented as picomoles of cyclic AMP per well (mean ± S.E.M.).

For the reverse transcriptase polymerase chain reaction (RT-PCR), 1321N1 wild-type and $P2Y_{11}$-transfected 1321N1 cell lines were cultured as described above. RNA was prepared ($n$ = 3) from $5 \times 10^6$ 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. First-strand cDNA was prepared from 5 $\mu$g of RNA using random hexamers and Superscript II reverse transcriptase in a 40-$\mu$l reaction volume according to the manufacturer's instructions. PCR reactions for the human $P2Y_2$, $P2Y_4$, and $P2Y_{11}$ employed the following primer pairs (forward, reverse) designed to amplify partial cDNAs from each sequence: P2Y2, 5′-GACTGCTAAAGCCAGCCTACGGGAC-3′, 5′-CCTGAAGTCCTCACTGCTGCCCAAC-3′, 409 bp; P2Y4, 5′-ACCCTATGGCTCTTCATCTTCCGCC-3′, 5′-AACAAGAGTGACCAGGCAGGGCACG-3′, 488 bp; and P2Y11, 5′-GGCTGGCGGCCTACAGAGCGTATAG-3′, 5′-CTCTGGGTTCCAGCTGTCCCTGTAG-3′, 442 bp. PCR reactions were performed (30 cycles of 94°C for 30 s; 65°C for 30 s, and 72°C for 30 s) using 1.25% (v/v) of each first-strand cDNA or 20 ng of human genomic DNA as a positive control with 2.5 units of Biotaq in a 25-$\mu$l amplification containing 1.5 mM $MgCl_2$ according to the manufacturer's instructions. Reverse transcriptions were also performed in the absence of the enzyme as a control for contaminating DNA and resulted in no amplification products when used in PCR, as did PCR reactions performed in the absence of added template. Amplicons (10 $\mu$l) were subjected to gel electrophoresis on a 2% (w/v) agarose gel.

Agonist purity was monitored by high-performance liquid chromatography analysis. A creatine phosphokinase regenerating system (10 mM creatine phosphate and 20 U/ml creatine phosphokinase), which maintains UTP and ATP concentrations, was included in some experiments. Data were analyzed using Prism v3.1 (GraphPad Software, San Diego, CA).

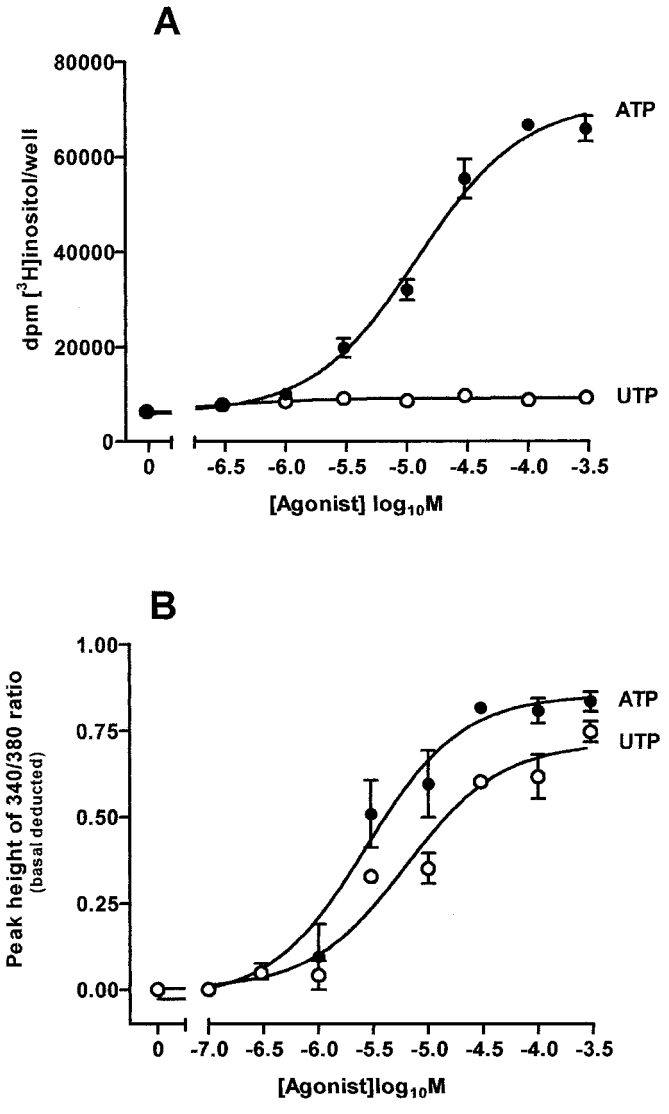


**Fig. 1.** Comparison of phosphoinositidase C and $Ca^{2+}$ responses to ATP and UTP. A, [$^3$H]$InsP_x$ responses to incubation with ATP and UTP. [$^3$H]Inositol-loaded cells were simulated for 20 min with ATP (●) or UTP (○) at the concentrations indicated, and [$^3$H]$InsP_x$ accumulation was measured. Data are mean ± S.E.M. from a single representative experiment. B, cytosolic $Ca^{2+}$ responses to ATP and UTP. Fura 2-loaded cells were perfused on a microscope stage and stimulated with ATP (●) or UTP (○) for 30 s at the concentrations shown; 340/380 ratios were recorded and basal deducted peak heights plotted from three separate experiments, each recorded from 8 to 10 cells.

## Results

[$^3$H]Inositol-labeled 1321N1 cells expressing hP2Y$_{11}$ receptors responded to increasing concentrations of ATP with a concentration-dependent increase in [$^3$H]InsP$_x$ accumulation (log $EC_{50}$ = −4.9 ± 0.13) as shown in Fig. 1A. UTP, over the same concentration range, gave no response, consistent with expectations from the literature (Fig. 1A).

Unexpectedly, however, fura-2-loaded 1321N1-hP2Y$_{11}$ cells stimulated with either ATP or UTP responded with similar concentration-dependent increases in $[Ca^{2+}]_c$ (Fig. 1B). Although within the same range, the concentration-response curves derived from these responses were significantly different ($P < 0.05$ by two-way ANOVA). The log $EC_{50}$ values for ATP and UTP were −5.57 ± 0.11 and −5.21 ± 0.10, respectively. The maximal response (340/380 ratio) to ATP (0.85 ± 0.03) was slightly greater than that to UTP (0.71 ± 0.03). We then conducted an equivalent series of experiments with ATP and UTP in the presence of a nucleotide triphosphate regenerating system. This served to purify the agonists by eliminating contaminating diphosphates and also to prevent interconversion during the stimulation period. The concentration-response curves were essentially unchanged (data not shown).

Figure 2 shows typical experimental traces from individual fura-2-loaded cells stimulated with ATP and UTP in the presence of a nucleotide triphosphate-regenerating system. The two nucleotides elicited similar increases in $[Ca^{2+}]_c$, with an immediate rapidly rising phase, followed by a plateau sustained for the duration of the agonist application. All cells were found to respond. The increase in pooled peak height with increasing agonist concentration depicted in Fig. 1B was caused by an increase in peak response of individual cells, not by recruitment of more cells, as seen in Fig. 2. Considerable overlap in the relative size of responses of individual cells to ATP and UTP was observed (Fig. 2). In control experiments, 1321N1 cells not expressing P2Y$_{11}$ receptors did not respond to nucleotides either with increases in $[Ca^{2+}]_c$ or inositol(poly)phosphates (data not shown).

In a further series of experiments, concentration-response curves were constructed to ATP and three derivatives: benzoyl ATP, ATPγS, and AR-C67085. As shown in Fig. 3, all were potent and full agonists. In [$^3$H]InsP$_x$ experiments, AR-C67085 was found to be the most potent agonist (data not shown). These results, together with the data presented in Fig. 1A, establish that the receptors expressed in these transfected 1321N1 cells have the characteristics of hP2Y$_{11}$ receptors.

To investigate whether the $[Ca^{2+}]_c$ increases elicited by ATP and UTP reflected mobilization from intracellular stores, cells were stimulated in the absence of added extracellular $Ca^{2+}$. Figure 4, A and B, show that in nominally $Ca^{2+}$-free medium, there was a substantial $Ca^{2+}$ response to both ATP and UTP. The response to ATP was significantly reduced ($P < 0.05$) when no extracellular $Ca^{2+}$ was added, whereas that to UTP was not significantly affected (by two-tailed unpaired $t$ test of responses from three separate experiments of 8–10 cells each). These results are consistent with mobilization of intracellular stores. In addition, they indicate that there is a substantial contribution by extracellular $Ca^{2+}$ to the response to ATP, but not to UTP.

To determine whether the maximal responses to ATP and UTP were additive, cells were stimulated with maximally effective concentrations of ATP and UTP (100 μM) either separately or simultaneously. As shown in Fig. 4C, the response to combined addition of ATP and UTP was indistinguishable from that to ATP alone. In a variation of this experimental design, cells were perfused with 100 μM ATP until the response had subsided (~4 min), and then UTP was added in the continued presence of ATP. Under these conditions, no response to 100 μM UTP was recorded (data not shown).

We then investigated whether these responses desensitize with repeated agonist administration. In these experiments, the stimulation period was extended to 4 min with intervals of 1 min between agonist applications. A typical result is shown in Fig. 5. The response to ATP was desensitized after

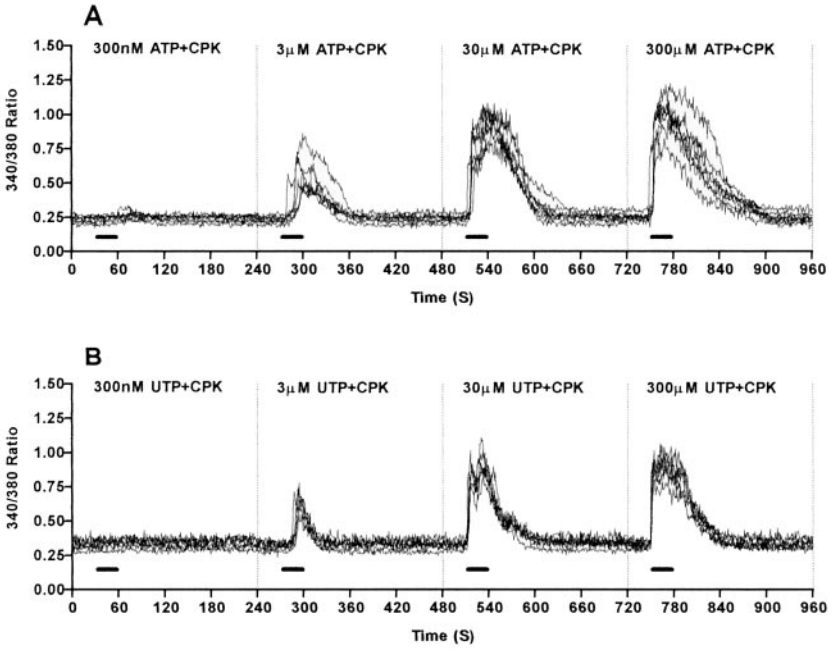


**Fig. 2.** Sample traces showing $Ca^{2+}$ responses to ATP (A) and UTP (B). Perfusion of Fura 2 loaded cells was in the presence of the CPK regeneration system, as described under *Materials and Methods*. Nucleotides were perfused for 30 s as indicated, at the concentrations shown. Each trace is the recording from a single cell.

one or more 4-min perfusions with this nucleotide. The response to UTP was also lost (Fig. 5A). However, after application of UTP, the response to ATP recovered. Figure 5B shows that this recovery was caused by time elapsed since the end of the last ATP perfusion (6 min) and was not dependent on the presence of UTP. Responses to UTP were also lost after a 4-min perfusion; however, this did not attenuate a subsequent response to ATP (Fig. 5C). When three applications of UTP were followed by a 6-min perfusion with buffer only, the UTP response did not recover (Fig. 5D). This contrasts with the result with ATP (Fig. 5B), which did recover under the same experimental conditions.

Pertussis toxin pretreatment was found to have differential effects on the $[Ca^{2+}]_c$ response of 1321N1-h$P2Y_{11}$ cells to ATP and UTP. As illustrated in Fig. 6, the concentration-response curve to ATP was unaffected by pertussis toxin pretreatment (100 ng.ml for 24 h). The concentration-response curves were not significantly different (two-way ANOVA), and the maximal response of pretreated cells was 97.8% of untreated cells. By contrast, the response to UTP was reduced by pertussis toxin. The maximal response was down to 43.2% of control value and the depressant effect on the concentration-response curve was highly significant ($P < 0.0001$ by two-way ANOVA, from three separate experiments with 8–10 cells in each).

2-APB has been shown to affect $Ca^{2+}$ responses involving $Ins(1,4,5)P_3$ receptors and store-operated $Ca^{2+}$ channels (Ma et al., 2000; van Rossum et al., 2000; Gregory et al., 2001). We investigated whether this compound had a differential effect on the $Ca^{2+}$ response to ATP and UTP. Figure 7, A and B, shows responses to ATP in the absence and presence of 2-APB (40 $\mu$M), respectively. The responses are unaffected by

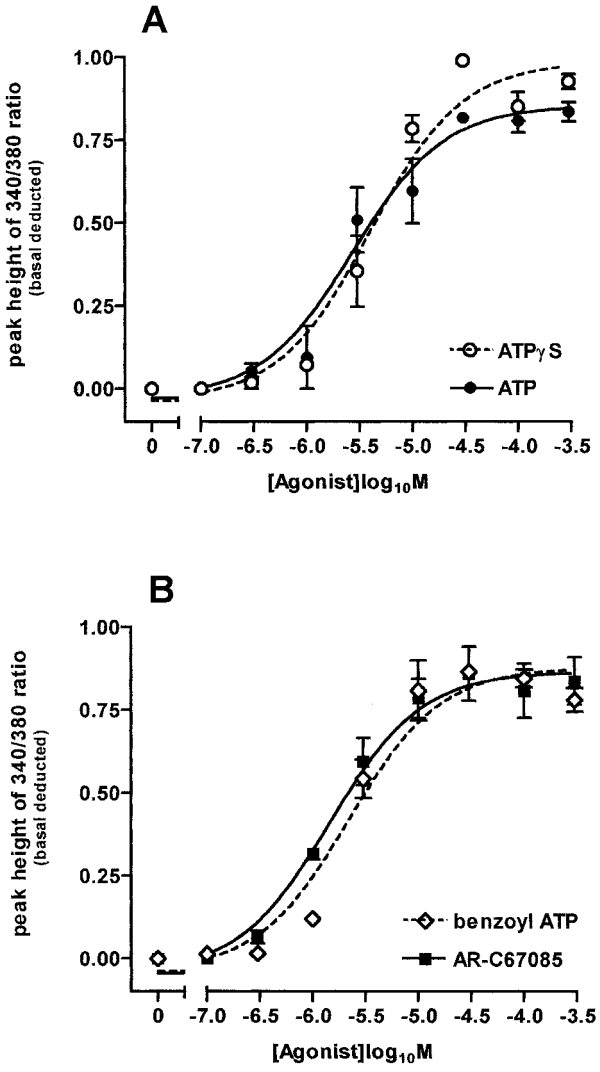


**Fig. 3.** Cytosolic $Ca^{2+}$ concentration response curves for ATP and ATP$\gamma$S (A) and benzoyl ATP and AR-C67085 (B). Fura 2-loaded cells were perfused on the microscope stage for 30 s with the concentrations of agonist shown. Data (mean ± S.E.M.) are peak basal deducted 340/380 responses pooled from three separate experiments each recorded from 8 to 10 cells.

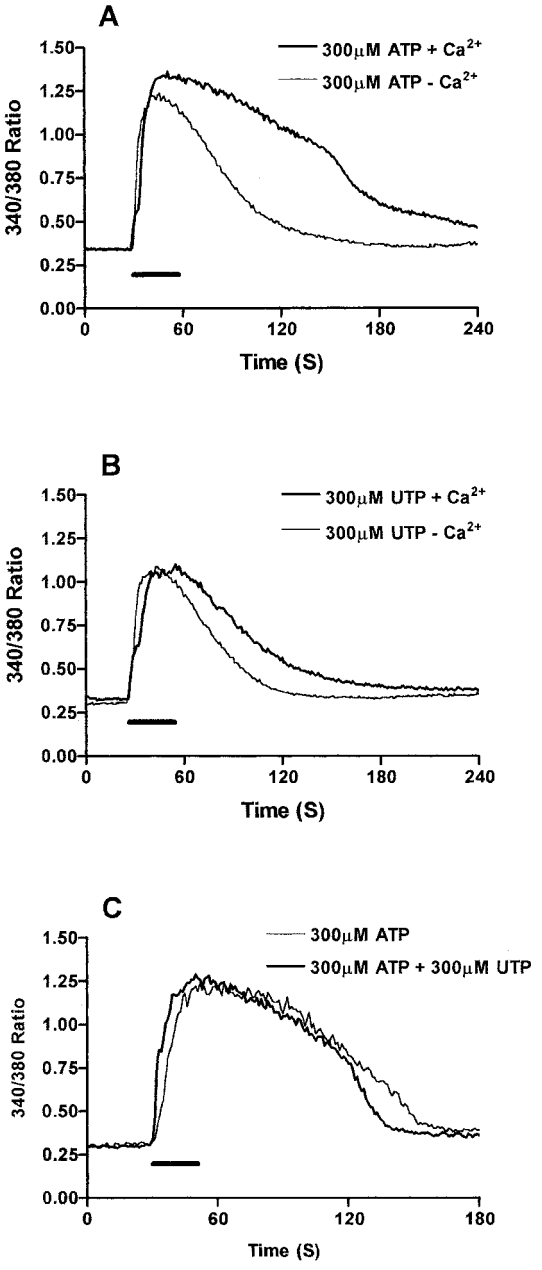


**Fig. 4.** A and B, cytosolic $Ca^{2+}$ traces on stimulation for 30 s with ATP and UTP, in the presence and absence of extracellular $Ca^{2+}$. Data are the mean traces from a total of 30 cells collected from three separate experiments. C, cytosolic $Ca^{2+}$ trace on stimulation with either ATP alone, or ATP and UTP added simultaneously, in the presence of extracellular $Ca^{2+}$. Pooled data from 10 cells in a single representative experiment.

2-APB. In contrast, the response to UTP (Fig. 7C) is substantially diminished by 2-APB (Fig. 7D). Pooled across three experiments measuring peak $Ca^{2+}$ responses (340/380 ratios), results were: ATP alone, 1.043 ± 0.036; ATP plus 2-APB, 0.985 ± 0.102; UTP alone, 0.914 ± 0.042; UTP plus 2-APB, 0.330 ± 0.062 (significantly different from UTP alone, $P < 0.001$, unpaired $t$ test).

To determine whether UTP stimulation of $hP2Y_{11}$ receptors regulates adenylyl cyclase, we measured cyclic AMP levels in cells in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine. Stimulations with nucleotides (300 $\mu$M) or forskolin (10 $\mu$M) were for 5 min at 37°C. Expressed as picomoles per well, results from three separate experiments were: control, 1.58 ± 0.26; ATP, 65.8 ± 19.7; forskolin, 15.9 ± 2.5; UTP, 1.78 ± 0.26; UTP with forskolin, 14.0 ± 0.29. The results confirm that ATP stimulates cyclic AMP accumulation. Contrasting with this UTP had no effect on either basal or forskolin-elevated cyclic AMP levels.

RT-PCR studies were undertaken to investigate the possibility that the $P2Y_{11}$-transfected 1321N1 cells also express mRNA transcripts for $P2Y_2$ or $P2Y_4$ receptors. RNA from the wild-type and $P2Y_{11}$-transfected cells was extracted and analyzed using primers specific for $hP2Y_{11}$, $hP2Y_2$, and $hP2Y_4$ encoding sequences. Genomic DNA was used to provide a positive control in these experiments. The results in Fig. 8 show that no P2Y-encoding transcripts were detected in the extracts from untransfected cells. In extracts from $P2Y_{11}$-expressing cells, there were no transcripts for either $P2Y_2$ or $P2Y_4$ receptors under conditions in which there was a very strong signal for the presence of $P2Y_{11}$ transcripts.

## Discussion

In this report, we show that both ATP and UTP are agonists at transfected $hP2Y_{11}$ receptors when the response measured is elevated $[Ca^{2+}]_c$. This is surprising given the considerable interest shown in $P2Y_{11}$ as an adenosine nucleotide-selective purinoceptor. Furthermore, we provide evidence that the two agonists, acting on one receptor population, increase $[Ca^{2+}]_c$ by different pathways. This may be

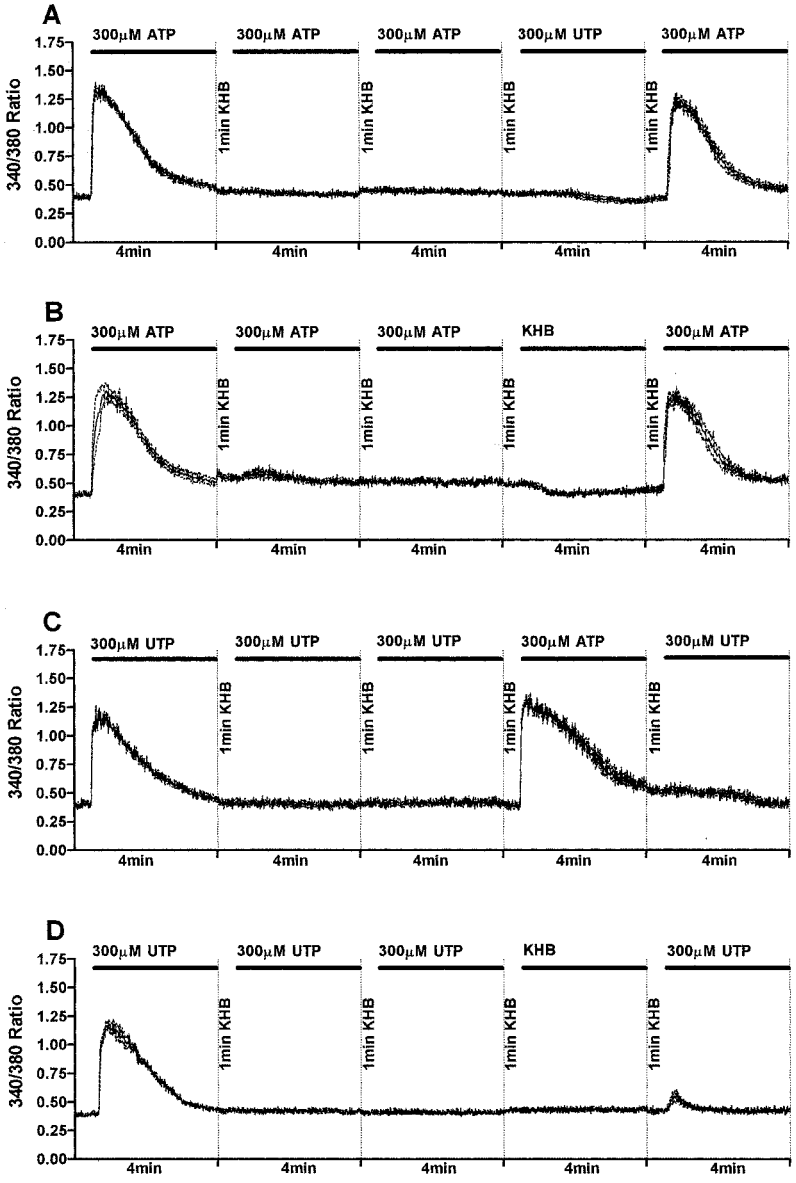


**Fig. 5.** Cytosolic $Ca^{2+}$ traces on repeated perfusion with ATP and UTP. Fura 2-loaded cells were perfused with ATP or UTP for the times indicated, with 1-min perfusion with KHB between applications. Data are the mean 340/380 ratios from 8 to 10 cells. Results are representative of those from four similar experiments.

caused by agonist-directed trafficking, in which the induction of agonist-specific receptor conformations activates two pathways from the same receptor, each leading to $Ca^{2+}$ mobilization and entry.

With respect to the first of these 2 issues, we are not aware of any published reports that describe the effect of UTP on $[Ca^{2+}]_c$ in cells heterologously expressing cloned $P2Y_{11}$ receptors. Here, we report a robust and reliable $[Ca^{2+}]_c$ response to UTP. To confirm that the response recorded was caused by UTP, some experiments were carried out in the presence of a nucleotide triphosphate-regenerating system. In addition, the $Ca^{2+}$ measurements were carried out using rapid perfusion of a low density of cells. Together these protocols effectively eliminate the involvement of agonist interconversion in the response. High-performance liquid chromatography analysis of agonist stocks also ruled out the involvement of any impurity. We therefore believe that the response measured is to UTP acting directly at the cell surface.

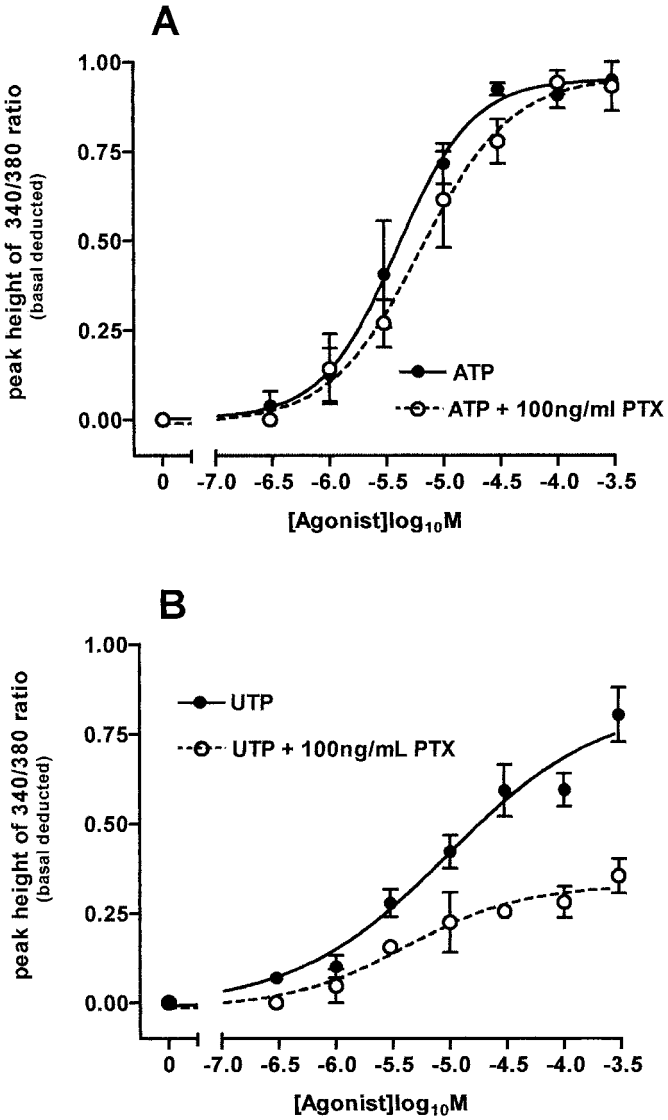


**Fig. 6.** Effect of pertussis toxin on cytosolic $Ca^{2+}$ responses to ATP (A) and UTP (B). Cells on coverslips were preincubated for 24 h in serum-free medium with or without pertussis toxin. Cells were loaded with fura 2 and perfused on the microscope stage with ATP or UTP at the concentrations shown for 30 s. Peak basal deducted 340/380 ratios were recorded and data presented as mean ± S.E.M. from three separate experiments, each recorded from 8 to 10 cells.

The data we report here are consistent with responses at transfected $P2Y_{11}$ receptors but not at other known P2Y receptors. For example, we found that UTP stimulation did not generate $[^3H]InsP_x$ in [$^3$H]inositol-loaded cells, and AR-C67085 was more potent than ATP in leading to accumulation of $[^3H]InsP_x$, consistent with results reported by Communi et al. (1997, 1999). When considering increases in $[Ca^{2+}]_c$, ATP, ATPγS, benzoyl ATP, and AR-C67085 were all found to be full and potent agonists. Although AR-C67085 is an antagonist at the $P2Y_{12}$ receptor, it displays agonist activity only at the $P2Y_{11}$ receptor. These data are inconsistent with the involvement of $P2Y_2$ receptors, at which UTP and ATP are equally effective at generating a phosphoinositidase C response. In addition, benzoyl ATP and AR-C67085 are not effective agonists at the $P2Y_2$ receptor. The data presented are also not consistent with $P2Y_4$ receptors, at which ATPγS is not an effective agonist (our unpublished data).

Our results cannot be explained by coexpression of other P2Y receptors induced in our cell cultures by the presence of $P2Y_{11}$ receptors. Not only is the pharmacology inconsistent with any known P2Y receptors apart from $P2Y_{11}$, as indicated above, but the increases in $[Ca^{2+}]_c$ in 1321N1 cells expressing P2Y receptors depend upon the activation of the phosphoinositidase C/Ins(1,4,5)$P_3$ pathway. If, for example, $P2Y_2$ were expressed alongside the $P2Y_{11}$ receptors in our cells, the $[Ca^{2+}]_c$ profiles might display some of the characteristics reported here, but there would also be a $[^3H]InsP_x$ response, as seen in parallel experiments with $P2Y_2$-transfected 1321N1 cells. To directly explore the possibility of $P2Y_2$ or $P2Y_4$ expression in these cells, we undertook RT-PCR studies, using primers for $P2Y_2$, $P2Y_4$, and $P2Y_{11}$, and found no transcripts for $P2Y_2$ and $P2Y_4$ under conditions that detect a very strong signal for $P2Y_{11}$ transcripts. We conclude, therefore, that these results cannot be explained by UTP acting at coexpressed $P2Y_2$ or $P2Y_4$ receptors.

UTP and ATP both generate $Ca^{2+}$ transients typical of a phosphoinositidase C-coupled receptor, with a rapid peak and slower decline toward baseline. However, the response to UTP was not significantly changed in nominally $Ca^{2+}$-free medium, compared with a substantial attenuation of the response to ATP. This indicates that the two agonists activate different signal transduction pathways. When ATP and UTP were applied together at a maximally effective concentration, the response was no greater than when one agonist was applied alone, consistent with the notion that the receptor has a single site at which the two agonists bind (Erb et al., 1995; Qi et al., 2001).

The possibility that ATP and UTP, acting on the $P2Y_{11}$ receptor, raise cytosolic $Ca^{2+}$ by different mechanisms is also suggested by the observation that ATP, but not UTP, stimulates detectable Ins(1,4,5)$P_3$ formation (current data; Communi et al., 1997). We cannot exclude the possibility that UTP activates a small and transient increase in Ins(1,4,5)$P_3$ synthesis that went undetected in these experiments. Alternatively, UTP may use an alternative pathway, such as cyclic adenosine 5′-diphosphoribose or the recently proposed NADPH pathway (Genazzani and Gallione, 1997; Churchill and Gallione, 2001). There have been previous reports of apparently inositol phosphate-independent increases in

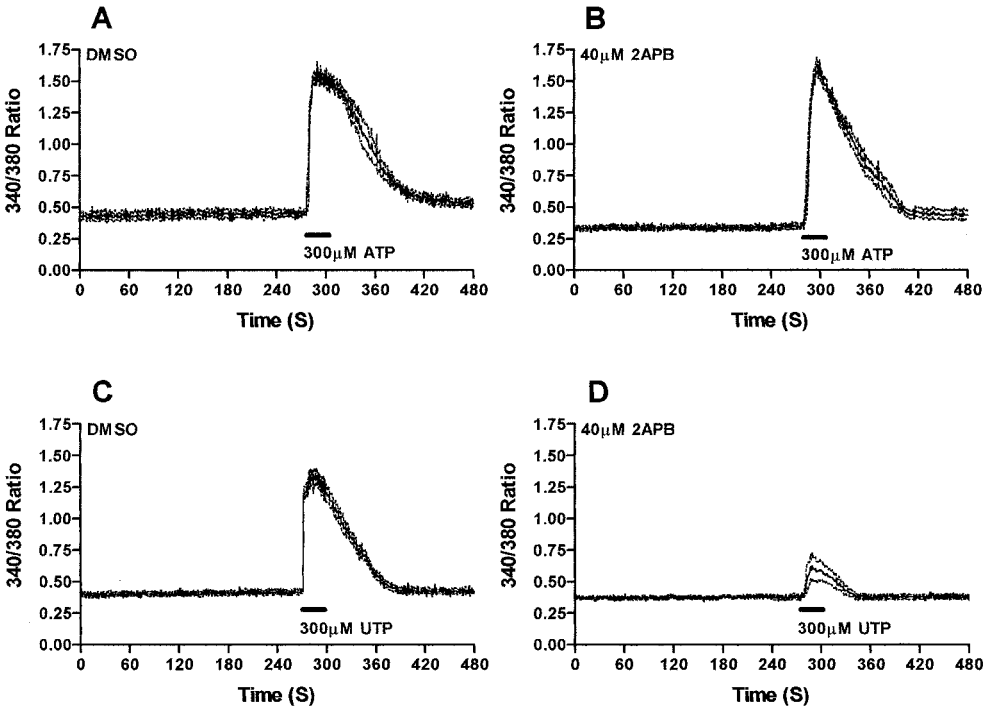


**Fig. 7.** Effect of 2-APB on cytosolic $Ca^{2+}$ responses to ATP and UTP. Fura 2-loaded cells were stimulated with ATP (A and B) and UTP (C and D) at the concentrations shown for 30 s. Either vehicle control (0.1% DMSO) or 2-APB (40 $\mu$M) was continuously perfused during the experimental period. Data are 340/380 ratios with each continuous trace representing the mean response from 8 to 10 cells. The results are typical of those from three identical experiments: pooled data across experiments are presented in the text.

$[Ca^{2+}]_c$ in response to activation of native P2Y receptors (e.g., Frelin et al., 1993; Albert et al., 1997; White et al., 2000).

The desensitization experiments are also consistent with two agonists acting at one receptor to generate different outcomes. The results illustrate that the UTP response is more sensitive to desensitization than is the response to ATP. One possible explanation is that ATP has a higher efficacy than UTP. The residual signal to the $Ca^{2+}$ response after desensitization may then be greater for ATP than for UTP and sufficient to generate a substantial response. However, it may be that the two agonists activate distinct pathways that vary in their ability to be down-regulated by desensitization protocols.

The experiments described here with pertussis toxin and 2-APB confirm that the two nucleotide agonists activate different signaling pathways to $Ca^{2+}$ mobilization. 2-APB was originally reported to be an effective antagonist at $Ins(1,4,5)P_3$ receptor channels (Ma et al., 2000; van Rossum et al., 2000). We therefore used this compound as a tool for investigating the involvement of $Ins(1,4,5)P_3$ receptors in the responses to ATP and UTP. Our hypothesis was that the $[Ca^{2+}]_c$ increase evoked by ATP would be blocked by 2-APB, whereas the UTP response, occurring in the absence of detectable inositol phosphate formation, would not. However, the reverse was true; the UTP response was blocked by 2-APB, and the ATP response was unaffected. The literature reveals complexities in the action of 2-APB. In cardiac myocytes, agonist-stimulated $Ins(1,4,5)P_3$-dependent $Ca^{2+}$ mobilization was unaffected by acute exposure to 2-APB (Gysembergh et al., 1999); in hepatocytes, 2-APB has been shown to leave $Ins(1,4,5)P_3$ mediated $Ca^{2+}$ mobilization intact but blocks vasopressin and thapsigargin-stimulated $Ca^{2+}$ influx (Gregory et al., 2001). A clear proposal for the mechanism of action of 2-APB in our experiments is difficult to formulate. Despite this, these results confirm that ATP and UTP elicit a $Ca^{2+}$ response by different signaling pathways.

The pertussis toxin experiments also provide a clear distinction between the 2 agonists: UTP is sensitive, but ATP is not, reflecting earlier reports in different cells of pertussis toxin sensitivity of UTP responses (e.g., Purkiss et al., 1994). This indicates that the G protein coupling of the $P2Y_{11}$ receptor is agonist-specific; when activated by UTP; the receptor is coupled to the $G_{i/o}$ family of G proteins. The observations on cyclic AMP accumulation are of interest here, because failure to stimulate basal levels or inhibit forskolin-stimulation suggests the UTP-activated receptor is not effectively coupled to either $G_s$ or $G_I$, so the pertussis toxin-sensitive $Ca^{2+}$ response is likely to be through $G_o$. When activated by ATP, the $Ca^{2+}$ response is pertussis toxin-insensitive, probably through $G_q$. Earlier work has shown that $P2Y_{11}$ receptors (when stimulated with ATP but not UTP) also couple to $G_s$ (Communi et al., 1997, 1999; Torres et al., 2002). Taken together, this leads to the interesting hypothesis that the $P2Y_{11}$ receptor may show agonist-directed promiscuity in coupling to three G proteins: $G_o$ coupling may occur with UTP stimulation, whereas ATP elicits receptor conformations that preferentially couple to $G_q$ and $G_s$.

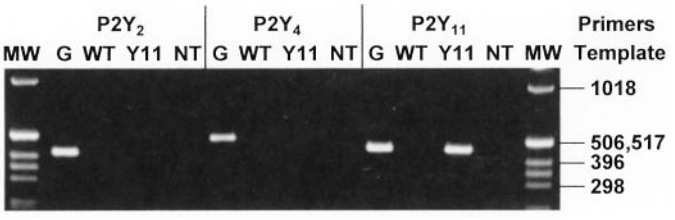


**Fig. 8.** RT-PCR of RNA extracts from untransfected (WT) and $P2Y_{11}$ expressing (Y11) 1321N1 cells using receptor-specific primers for $P2Y_2$ (left), $P2Y_4$ (middle), and $P2Y_{11}$ (right). Also shown are results of amplification of genomic DNA as template (G) forming a positive control, and amplification with no template (NT) as a negative control. The results shown are from a single experiment of three undertaken, each with an identical outcome.

Kenakin (1995) has pointed out that the two explanations for differential activation of signaling pathways by different agonists are different strengths of signal or agonist trafficking. In the former explanation, a high-efficacy agonist may have the potential to activate both tightly and loosely coupled G proteins, whereas a low-efficacy agonist will activate only the more efficiently coupled G proteins. The result will be differential recruitment of signaling pathways, not because of distinct receptor conformations, but because the lower efficacy agonist produces insufficient activated receptor to produce a significant response from the less efficiently coupled G protein. This promiscuity is fundamentally different from agonist trafficking with agonist-specific receptor conformations, each of which may show fidelity in coupling to G proteins. In this article, we have presented clear evidence for agonist-specific signaling at the $P2Y_{11}$ receptor, but our results do not permit a clear distinction between these two explanations for the differential activation of $P2Y_{11}$ receptors by ATP and UTP.

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