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UTP is not a biased agonist at human P2Y<sub>11</sub> receptors

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**Abbreviations**

1321N1-hP2Y<sub>2</sub> cells - 1321N1 cells stably expressing the human P2Y<sub>2</sub> receptor

1321N1-hP2Y<sub>11</sub> cells - 1321N1 cells stably expressing the human P2Y<sub>11</sub> receptor

95% cl - 95% confidence limits

ATP - adenosine 5'-triphosphate

GPCR - G protein-coupled receptors

IPs - inositol phosphates

UTP - uridine 5'-triphosphate

## **Abstract**

Biased agonism describes a multi-state model of G protein-coupled receptor activation in which each ligand induces a unique structural conformation of the receptor, such that the receptor couples differentially to G proteins and other intracellular proteins. P2Y receptors are G protein-coupled receptors that are activated by endogenous nucleotides, such as ATP and UTP. A previous report suggested that UTP may be a biased agonist at the human P2Y<sub>11</sub> receptor, as it increased cytosolic [Ca<sup>2+</sup>], but did not induce accumulation of inositol phosphates, whereas ATP did both. The mechanism of action of UTP was unclear, so the aim of this study was to characterise the interaction of UTP with the P2Y<sub>11</sub> receptor in greater detail. Intracellular Ca<sup>2+</sup> was monitored in 1321N1 cells stably expressing human P2Y<sub>11</sub> receptors using the Ca<sup>2+</sup>-sensitive fluorescent indicator, fluo-4. ATP evoked a rapid, concentration-dependent rise in intracellular Ca<sup>2+</sup>, but surprisingly, even high concentrations of UTP were ineffective. In contrast, UTP was slightly, but significantly more potent than ATP in evoking a rise in intracellular Ca<sup>2+</sup> in 1321N1 cells stably expressing the human P2Y<sub>2</sub> receptor, with no difference in the maximum response. Thus the lack of response to UTP at hP2Y<sub>11</sub> receptors was not due to a problem with the UTP solution. Furthermore, coapplying a high concentration of UTP with ATP did not inhibit the response to ATP. Thus contrary to a previous report, we find no evidence for an agonist action of UTP at the human P2Y<sub>11</sub> receptor, nor does UTP act as an antagonist.

**Keywords:** Biased agonism, P2Y<sub>11</sub> receptor, inositol phosphates, intracellular Ca<sup>2+</sup>

## Introduction

In the classical pharmacological model of drug-receptor interaction, all agonists at a G protein-coupled receptor (GPCR) activate the same G protein(s) and signalling pathways and agonists differ only in their affinity for the receptor and efficacy in stimulating the second messenger(s). It is now clear, however, that receptors can in fact couple to different G proteins and signalling pathways in an agonist-dependent manner, a process termed biased agonism or functional selectivity [1]. For example, coupling of the D<sub>2</sub>-dopamine receptor to G $\alpha_{i1}$ , G $\alpha_{i2}$ , G $\alpha_{i3}$  and G $\alpha_{o1}$  varies with the agonist used [2] and the relative efficacy of 5-HT<sub>2C</sub> agonists depends on whether phospholipase C or A<sub>2</sub> activity is measured [3]. Similar results have been reported for a variety of other GPCR, including  $\mu$ -opioid, adrenoceptors and vasopressin receptors.

These data suggest a multi-state model of GPCR activation in which each ligand induces a unique structural conformation of the receptor that couples differentially to G proteins and other proteins, such as arrestins, that interact with GPCR. Direct evidence for this model was obtained recently by imaging agonist-induced changes in the conformational structure of the  $\beta_2$ -adrenoceptor [4] and arginine-vasopressin type 2 receptor [5]. In both studies one group of agonists caused the cytoplasmic end of helix VI of the receptor to move, shifting the equilibrium towards G protein coupling, whilst another group moved the cytoplasmic end of helix VII, leading to binding of  $\beta$ -arrestin to the receptor.

P2Y receptors are activated by endogenous nucleotides, such as adenosine 5'-triphosphate (ATP) and uridine 5'-triphosphate (UTP) [6-8], but there are few reports of biased agonism. ATP and UTP induce differential interaction of the human P2Y<sub>2</sub> receptor with  $\beta$ -arrestins and the downstream increase in phosphorylation of ERK elicited by UTP was transient, whereas the response to ATP was prolonged [9]. Intriguingly, it has been reported that whilst ATP stimulation of the human P2Y<sub>11</sub> receptor caused accumulation of inositol phosphates (IPs) and a rise in cytosolic [Ca<sup>2+</sup>], UTP had only the latter effect [10]. Thus ATP and UTP appear to interact with the P2Y<sub>11</sub> receptor to recruit distinct signalling pathways, but how UTP produced its effects is unclear. Biased agonism has important implications for rational drug design as it raises the possibility of developing new drugs that only activate a desired, beneficial function of a GPCR, thus optimising their therapeutic effect. The aim of this study was, therefore, to investigate the mechanisms by which UTP increases cytosolic [Ca<sup>2+</sup>] and so characterise biased agonism at P2Y<sub>11</sub> receptors in greater detail.

## Methods and Materials

### Culture of 1321N1 cells

1321N1 cells, a human astrocytoma cell line, stably expressing the human P2Y<sub>11</sub> (1321N1-hP2Y<sub>11</sub>) [11] or P2Y<sub>2</sub> (1321N1-hP2Y<sub>2</sub>) [12] receptors were maintained in 5% CO<sub>2</sub>, 95% O<sub>2</sub> in a humidified incubator at 37°C, in Dulbecco's Modified Eagles Media (DMEM) supplemented with 10% foetal calf serum, 1% non-essential amino acids, 1% penicillin (10,000 units/ml) and streptomycin (10 mg/ml). For recording intracellular Ca<sup>2+</sup>, the cells were plated onto 13 mm glass coverslips coated with poly-L-lysine (0.1 mg/ml). Experiments were performed once a confluent monolayer of cells had developed.

### Ca<sup>2+</sup> imaging

Intracellular Ca<sup>2+</sup> was monitored using the Ca<sup>2+</sup>-sensitive fluorescent indicator, fluo-4. Cells on a coverslip were incubated with fluo-4 AM ester (5 µM) at room temperature in the dark for at least 1 hr. The coverslip was then placed in the recording chamber of a Perkin Elmer LS50B luminescence spectrophotometer and the cells superfused continuously at 4ml/min and room temperature with buffer composed of (mM): NaCl 122; KCl 5; HEPES 10; KH<sub>2</sub>PO<sub>4</sub> 0.5; NaH<sub>2</sub>PO<sub>4</sub> 0.5; MgCl<sub>2</sub> 1; glucose 11; CaCl<sub>2</sub> 1.8, titrated to pH 7.3 with NaOH. Fluo-4 fluorescence, measured as arbitrary units (AU), in a population of cells was sampled at 10 Hz following stimulation at 494±10 nm and the emission recorded at 516±10 nm using FL Winlab software (V4.00.02). ATP and UTP were added in the superfusate for 90-120 sec at 10 min intervals. Preliminary experiments showed that reproducible responses were evoked under these conditions. Unless indicated otherwise, data were normalised by calculating the response in AU as a percentage of the response to ATP (10 µM) (1321N1-hP2Y<sub>11</sub> cells) or ATP (1 µM) (1321N1-hP2Y<sub>2</sub> cells).

### IP formation

Generation of inositol phosphates (IPs) was measured as described previously [11]. Briefly, 1321N1-hP2Y<sub>11</sub> cells were seeded in 24-well plates at 10<sup>5</sup> cells per well and assayed 3 days later when confluent. Inositol lipids were radiolabeled by incubation of the cells for 24 h with 200 µl inositol-free, serum-free DMEM high glucose and 0.4 µCi *myo*-[<sup>3</sup>H]inositol. No changes of medium were made subsequent to the addition of [<sup>3</sup>H]inositol. Drugs were added in 50 µl of a 5-fold concentrated solution in 50 mM LiCl, 250 mM HEPES, pH 7.4. Following a 5 min incubation at 37°C, the medium was aspirated and the assay terminated by adding 0.75 ml boiling 10 mM EDTA, pH 8.0. [<sup>3</sup>H]IPs were then resolved by Dowex AG1-X8 columns [13].

## Data analysis

Values in the text and figures refer to mean  $\pm$  S.E.M. or geometric mean with 95% confidence limits (95% cl) for EC<sub>50</sub> values. Data were compared by paired (Ca<sup>2+</sup> imaging data) and unpaired (IP data) t-tests as appropriate. Differences were considered significant if P<0.05.

## Drugs, solutions

ATP (disodium salt) and UTP (sodium salt) (Sigma/RBI, UK) were dissolved in deionised water as 10 mM stock solutions and diluted in buffer before use. Fluo-4 AM ester (Invitrogen, UK) was dissolved in DMSO as a 1 mM stock solution, frozen immediately and stored at -20°C. On the day of use it was diluted in buffer before use.

## Results

A previous study reported that ATP and UTP both increased intracellular Ca<sup>2+</sup> in 1321N1-hP2Y<sub>11</sub> cells [10], so initial experiments were carried out to confirm these data. Figure 1 shows that ATP (0.1 - 30  $\mu$ M) evoked a rapid rise in intracellular Ca<sup>2+</sup> that was not maintained in the continued presence of ATP. The response amplitude was concentration-dependent, with an EC<sub>50</sub> = 2.1  $\mu$ M (95% cl. 1.8 - 2.4  $\mu$ M) (n=5). Surprisingly, in the same cells, even high concentrations of UTP (10  $\mu$ M, n= 6 and 100  $\mu$ M, n=12) had no effect on intracellular Ca<sup>2+</sup> (Figure 1).

To confirm that the UTP solution was active, we repeated these experiments in 1321N1-hP2Y<sub>2</sub> cells. Both nucleotides evoked rapid, transient rises in intracellular Ca<sup>2+</sup> in a concentration-dependent manner, with EC<sub>50</sub> values of 73 nM for UTP (95% cl. 59 - 90 nM) and 176 nM for ATP (95% cl. 156 - 199 nM) (n=4 each) (Figure 2). Indeed, UTP was slightly, but significantly more potent than ATP (P<0.01), but there was no difference in the maximum response. The lack of response to UTP in 1321N1-hP2Y<sub>11</sub> cells was not, therefore, due to a problem with the UTP solution.

These data show that we could not confirm the previous report [10] that UTP acts at the hP2Y<sub>11</sub> receptor to raise intracellular Ca<sup>2+</sup>. A possible explanation is that UTP did bind to the receptor, but for an unknown reason, did not activate it. To address this possibility we determined if coadministration of UTP inhibited the response to ATP. Control responses were obtained to ATP (2  $\mu$ M), a concentration close to its EC<sub>50</sub>. Superfusing 1321N1-hP2Y<sub>11</sub> cells with UTP (100  $\mu$ M) for 10 min before reapplying ATP (2  $\mu$ M) along with UTP (100  $\mu$ M) had no significant effect on the response to ATP (Figure 3a,b).

Previous studies on hP2Y<sub>11</sub> receptors reported that ATP, but not UTP, induced accumulation of IPs [14,15], so we determined if UTP could inhibit this action of ATP. UTP (100 μM) alone did not alter basal level of IPs (not shown) nor was the rise in IPs elicited by ATP (3 μM) significantly affected by coadministration of UTP (100 μM) (Figure 3c). Thus two bioassays of receptor activity show that UTP is also not an antagonist at the human P2Y<sub>11</sub> receptor.

## Discussion

Biased agonism, a process in which agonists bind the same receptor subtype, but activate different signalling pathways, is an important discovery that has implications for rational drug design, as it could lead to the development of new therapeutic agents that selectively stimulate beneficial functions of a GPCR. A previous study indicated that UTP is a biased agonist at human P2Y<sub>11</sub> receptors, inducing Ca<sup>2+</sup> mobilisation, so the aim of this study was characterise this action in detail. We were, however, unable to replicate UTP agonism. Nucleotides can also act as P2Y receptor antagonists [16], but even high concentrations of UTP did not inhibit ATP in two bioassays of P2Y<sub>11</sub> receptor activity, Ca<sup>2+</sup> mobilisation and accumulation of IPs. Thus we conclude that UTP is neither an agonist nor an antagonist at the human P2Y<sub>11</sub> receptor.

In this study, ATP evoked a rapid, concentration-dependent rise in intracellular Ca<sup>2+</sup> with an EC<sub>50</sub> of 2.1 μM, which is very close to the value of 2.7 μM seen in the earlier study that suggested biased agonism at the P2Y<sub>11</sub> receptor [10]. We reported previously an EC<sub>50</sub> of 8.1 μM for ATP-induced accumulation of IPs in the same cells [11], which is similar to the value of 12.6 μM reported in the earlier study [10] for that response. Thus the potency of ATP seen in the two laboratories was essentially the same, suggesting similar levels of P2Y<sub>11</sub> receptor expression. This is not surprising, as the 1312N1-hP2Y<sub>11</sub> cell line used in both studies was generated by two of the present authors (CK, RAN) at the University of North Carolina. Despite this, we did not observe a rise in intracellular Ca<sup>2+</sup> when UTP was applied, even at 100 μM, a concentration that was almost maximally effective in the earlier study [10]. 1321N1 cells are often used for expression of recombinant P2Y receptors because they not express any endogenous P2Y subtypes [6] and the earlier study [10] confirmed the absence of mRNA for the UTP-sensitive P2Y<sub>2</sub> and P2Y<sub>4</sub> receptors in the 1321N1-hP2Y<sub>11</sub> cells, eliminating those subtypes as the site of action of UTP. Thus it is unlikely that the inactivity of UTP in our study was due to differences in the expression of P2Y receptors.

Several other possible explanations for the difference in the responsiveness to UTP can be considered. Contamination by hydrolysis products of UTP is unlikely to be the cause of the discrepancy as the previous study monitored UTP purity by HPLC and eliminated its initial

breakdown product, uridine 5'-diphosphate, using a creatine phosphokinase regenerating system. In contrast, the UTP solution used in the present study was  $\geq 80\%$  pure, with  $\leq 15\%$  UDP and  $\leq 5\%$  UMP, so if hydrolysis products of UTP were to induce a  $\text{Ca}^{2+}$  response, it would most likely have been seen in our study. Differences in the composition of the extracellular buffer are also unlikely to be an explanation, as in both studies the 1321N1-hP2Y<sub>11</sub> cells were superfused with a Krebs-HEPES buffer containing 10 mM HEPES. Although White et al., (2003) gave no further details of the buffer composition, any differences in the ions present would have to be substantial in order to produce such a large difference in the responsiveness to UTP. Consequently, the most feasible explanation in our view is contamination of the UTP solution by a non-nucleotide agent acting at a receptor that is not a P2Y receptor.

In conclusion, we have demonstrated that ATP acts at the human P2Y<sub>11</sub> receptor to initiate a rise in cytoplasmic  $\text{Ca}^{2+}$ , but this response is neither mimicked nor inhibited by high concentrations of UTP. Thus contrary to a previous report, we have found no evidence that UTP is a biased agonist at the human P2Y<sub>11</sub> receptor.

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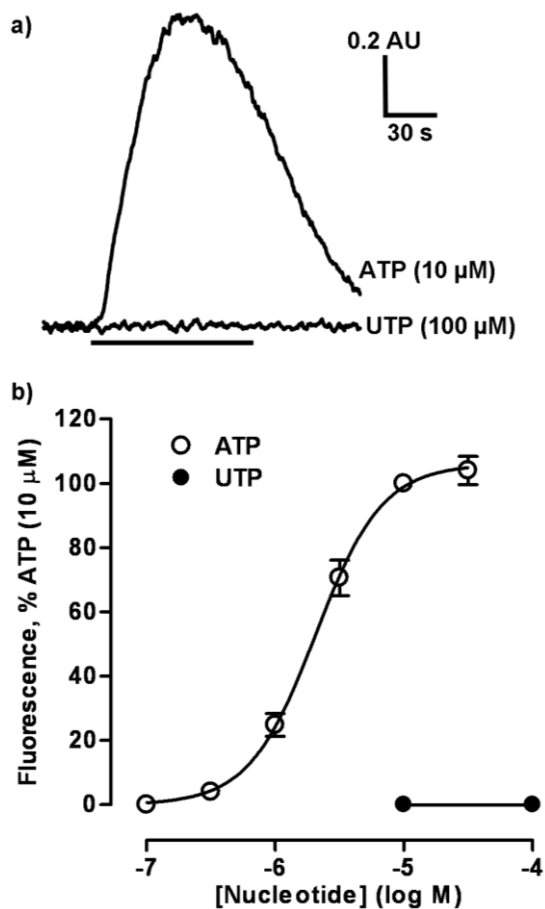


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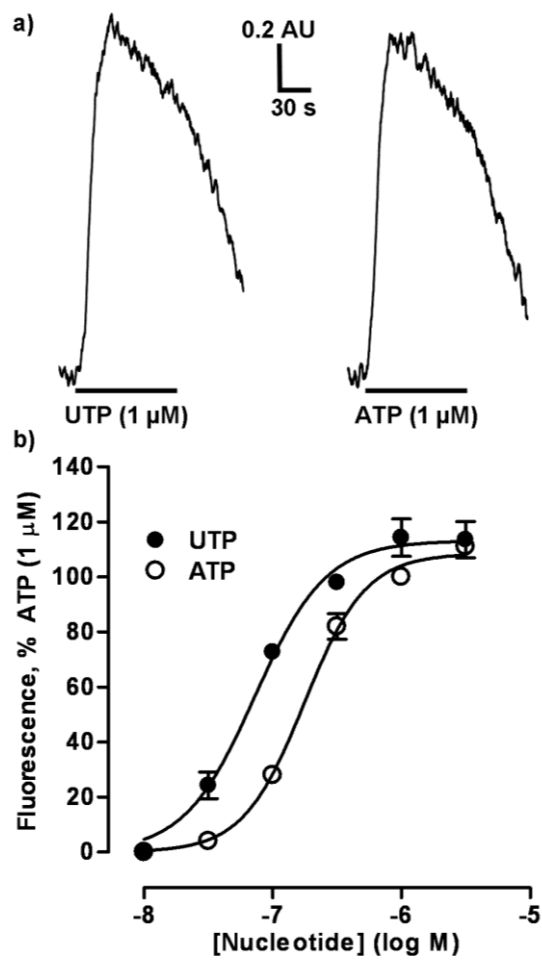
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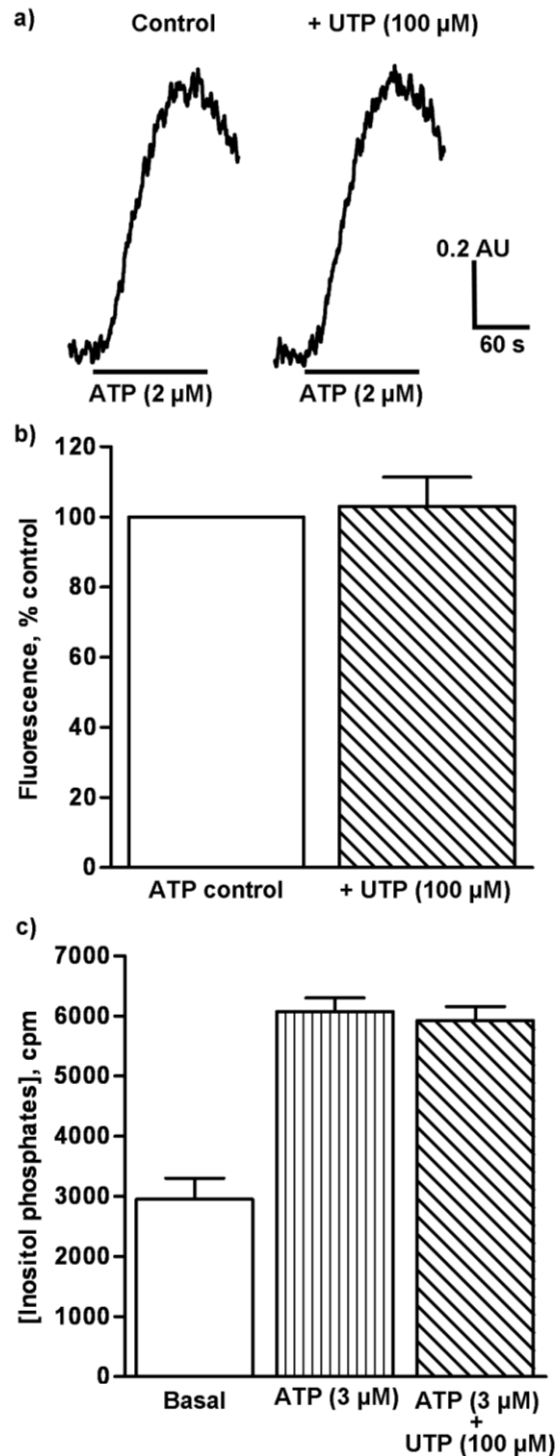
**Fig. 1 ATP, but not UTP increases intracellular  $\text{Ca}^{2+}$  in 1321N1-hP2Y<sub>11</sub> cells.**

a) The superimposed traces show fluo-4 fluorescence during superfusion with ATP (10 μM) (upper trace) and UTP (100 μM) (lower trace) for 90 sec, as indicated by the horizontal bar. Both records are from the same population of cells. b) The mean peak amplitude of responses evoked by ATP (n=5) and UTP (10 μM, n= 6 and 100 μM, n=12) are shown. Responses are expressed as % of the response to ATP (10 μM). Vertical lines show S.E.M.



**Fig. 2 ATP and UTP increase intracellular  $\text{Ca}^{2+}$  in 1321N1-hP2Y<sub>2</sub> cells.**

a) The trace shows fluo-4 fluorescence during superfusion with UTP (1 μM) for 90 sec, as indicated by the horizontal bar. b) The mean peak amplitude of responses evoked by ATP (n=4) and UTP (n=4), expressed as % of the response to ATP (1 μM). Vertical lines show S.E.M.



**Fig. 3 UTP does not inhibit ATP-evoked responses in 1321N1-hP2Y<sub>11</sub> cells.**

a) The traces show the rise in intracellular Ca<sup>2+</sup> evoked by ATP (2 μM) in the same population of cells before (left-hand side) and after superfusion for 10 min with UTP (100 μM) (right-hand side), as indicated by the horizontal bars. b) The mean peak amplitude of responses evoked by ATP (2 μM) in the absence and presence of UTP (100 μM, n= 5) are shown. Responses are expressed as % of the control response to ATP (2 μM). c) The mean basal level of IP<sub>3</sub> (left-hand column) and the mean amplitude of responses evoked by ATP (3 μM) in the absence (middle column) and presence of UTP (100 μM) (right-hand column) are shown. n=3. Vertical lines indicate S.E.M.