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Pharmacological characterization of the human P_{2Y4} receptor

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Abstract

The $P2Y_4$ receptor is a new member of the P2Y family which functionally behaves as a pyrimidinergic receptor. The pharmacological properties of the human $P2Y_4$ receptor have been characterized following its stable expression in 1321N1 astrocytoma cells. UTP induced a biphasic accumulation of inositol trisphosphates, with an early peak at 30 s followed by a smaller but more sustained accumulation. ATP was a pure antagonist at early times and later behaved as a partial agonist. At 20 min, the rank order of potency of various nucleotides was the following: UTP > UDP = deoxyUTP > 5-bromo-UTP > ITP > ATP. Diadenosine polyphosphates also stimulated the production of inositol trisphosphates (after 20 min), more potently than ATP, but their maximal effect represented only 20–25% of that of UTP. Pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid inhibited strongly the UTP response, whereas suramin was inactive and reactive blue 2 had an intermediate effect. Pertussis toxin inhibited the response to UTP at early times ($62 \pm 5\%$ inhibition at 30 s), but its effect was no longer observed at 5 or 20 min. It is speculated that the $P2Y_4$ receptor can exist in two distinct activation states differing in terms of time-course, specificity for uridine nucleotides and G-protein coupling.

Keywords: Uridine nucleotide receptor; UTP; Inositol trisphosphate

1. Introduction

The cloning of several receptors for ATP has been reported since 1993, leading to a complete refoundation of the nomenclature of P_2 receptors (Fredholm et al., 1994). They are now subdivided into two classes: G-protein-coupled heptahelical receptors called P2Y receptors and receptors with intrinsic ion channel activity or P2X receptors. At least seven members of the P2Y family have been cloned so far. The existence of some of these subtypes was predicted from previous pharmacological studies. The $P2Y_1$ receptor, corresponding to the P2Y receptor of the former pharmacological classification, has been cloned in several species from chicken to man and is characterized by the high affinity of 2-methylthio derivatives of ATP and ADP (Webb et al., 1993; Filtz et al., 1994; Henderson et al., 1995; Tokoyama et al., 1995; Ayyanathan et al., 1996). The $P2Y_2$ receptor is equivalent to the former P_{2U} receptor: the mouse (Lustig et al., 1993; Erb et al., 1993), rat (Rice et al., 1995) and human (Parr et al., 1994) receptors have been cloned and are characterized by the equipotency of ATP and UTP. The cloning of the other P2Y receptors could not have been anticipated from previous functional studies. The $P2Y_3$ receptor cloned from chick brain has not yet been fully described in the literature (Barnard et al., 1994). The $P2Y_5$ receptor, previously called 6H1, has been isolated from activated chicken lymphocytes and unlike the others does not seem to be coupled to phospholipase C (Kaplan et al., 1993; Webb et al., 1996). Finally, the $P2Y_4$ (Communi et al., 1995a; Nguyen et al., 1995) and $P2Y_6$ (Chang et al., 1995) receptors are unique in that they have a preference for uridine over adenine nucleotides. The human $P2Y_4$ receptor has been partially characterized following its stable expression in 1321N1 human astrocytoma cells: Communi et al. (1995a) measured the accumulation of inositol trisphosphates ($InsP_3$), whereas Nguyen et al. (1995) relied mostly on the spectrofluorimetric measurement of cytoplasmic Ca^{2+}. From these two studies, it is clear that the $P2Y_4$ receptor is a phospholipase C-coupled receptor for uridine nucleotides. There were, however, some discrepancies, in particular concerning the true activity of ATP, which was a pure antagonist in the study of

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Nguyen et al. (1995), but behaved like a partial agonist according to Communi et al. (1995a). The purpose of the present study was to resolve this apparent contradiction and to provide a full characterization of the human $P2Y_4$ receptor in terms of time-course, rank order of potency of a range of nucleotides, sensitivity to antagonists and coupling to G-proteins.

2. Materials and methods

2.1. Materials

Trypsin was from Flow Laboratories (Bioggio, Switzerland) and the culture media, fetal calf serum and G418 were purchased from Life Technologies (Merelbeke, Belgium). Myo-D-[2-^{3}H]inositol (17.7 Ci/mmol) and [α^{32}P]UTP (3000 Ci/mmol) were from Amersham (Ghent, Belgium). Dowex AG1X8 (formate form) was from Bio-Rad Laboratories (Nazareth Eke, Belgium). ATP, UTP, UDP, 5-bromo-UTP (5BrUTP), deoxyUTP (dUTP), ITP, the diadenosine polyphosphates (AP_3A, AP_4A, AP_5A and AP_6A) and pertussis toxin were from Sigma (St. Louis, MO, USA). Suramin, reactive blue 2 and pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) were from Research Biochemicals (Natick, MA, USA).

2.2. Expression of the human $P2Y_4$ receptor into 1321N1 human astrocytoma cells

1321N1 cells were transfected with the human $P2Y_4$ coding sequence inserted into the pcDNA3 expression vector using the calcium phosphate precipitation method as described (Communi et al., 1995a; Velu et al., 1989). Individual clones were isolated by limiting dilution from the pool of 1321N1 cells selected with G418 (400 μg/ml). Unless noted, the experiments were performed with the clone 11 chosen for its large $InsP_3$ response to UTP.

2.3. Measurement of $InsP_3$ production

1321N1 cells were labeled for 24 h with 10 μCi/ml [^{3}H]inositol in inositol-free DMEM (Dulbecco's modified Eagle's medium) medium containing 5% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 400 μg/ml G418. Cells were washed twice with KRH (Krebs-Ringer HEPES) buffer of the following composition (124 mM NaCl, 5 mM KCl, 1.25 mM $MgSO_4$, 1.45 mM $CaCl_2$, 25 mM HEPES (pH 7.4) and 8 mM glucose) and incubated in this medium for 30 min. The agonists were added in the presence of LiCl (10 mM) and the incubation was stopped after 30 s, 5 min or 20 min by the addition of an ice-cold 3% perchloric acid solution. For the time-course study, LiCl (10 mM) was added 5 min before the agonists and the incubation was stopped at different times. When tested, pertussis toxin (20 ng/ml) was added for 18 h during the labeling period and during the stimulation by the agonist. Inositol phosphates were extracted and $InsP_3$ was isolated by chromatography on Dowex column as described previously (Communi et al., 1995b). The EC_{50} values were determined by curve fitting (Sigma Plot: version 2.0).

2.4. Radioligand binding assay

Binding assays of [α-^{32}P]UTP to cell membranes were carried out in Tris-HCl (50 mM, pH 7.5), 1 mM EDTA in a final volume of 0.5 ml, containing 25–50 μg of protein and 0.5 nM of radioligand (Motte et al., 1996). The assays were conducted at 30°C for 5 min. Incubations were stopped by the addition of 4 ml of ice-cold Tris-HCl (50 mM, pH 7.5) and rapid filtration through Whatman GF/B filters under reduced pressure. The filters were then washed three times with 2 ml of the same ice-cold Tris-HCl buffer. Radioactivity was quantified by liquid scintillation counting, after an overnight incubation of the filters in liquid scintillation mixture.

3. Results

A preliminary functional characterization of the $P2Y_4$ receptor has been performed recently after stable transfection into 1321N1 astrocytoma cells: as others (Filtz et al., 1994), we have chosen to determine the accumulation of $InsP_3$ after 20-min incubation with the agonists in the presence of 10 mM LiCl (Communi et al., 1995a), whereas Nguyen et al. (1995) measured the $[Ca^{2+}]_i$ peak obtained around 5 s after agonist addition. Depending on the method, ATP behaved as a partial agonist (Communi et al., 1995a) or as a pure antagonist (Nguyen et al., 1995). In fact, the discrepancy in ATP responsiveness between the two studies might be a matter of kinetics. Indeed, we observed that the response to UTP was biphasic, with a peak reached at 30 s, followed by a more sustained stimulation of lower magnitude (Fig. 1A). With ATP, only that second phase was detectable: its effect became apparent after 1 min of stimulation only and was stable for at least 20 min (Fig. 1A,B). As for UTP, the stimulation by UDP was biphasic, but it was slightly delayed (Fig. 1A,B). Inclusion of LiCl had little effect on the initial peak induced by UTP or UDP, but it strongly enhanced the following plateau phase (Fig. 1B).

As reported previously (Communi et al., 1995a), the maximal effect of ATP observed after a 20-min incubation represented about 27 ± 9% of that of UTP (mean ± S.D. of 10 experiments). In order to demonstrate that ATP is able to antagonize the UTP response, incubations of 1321N1 cells were conducted with ATP alone or in combination with UTP. Fig. 2 shows that at high concentration (500 μM or more), ATP was able to inhibit the effect of

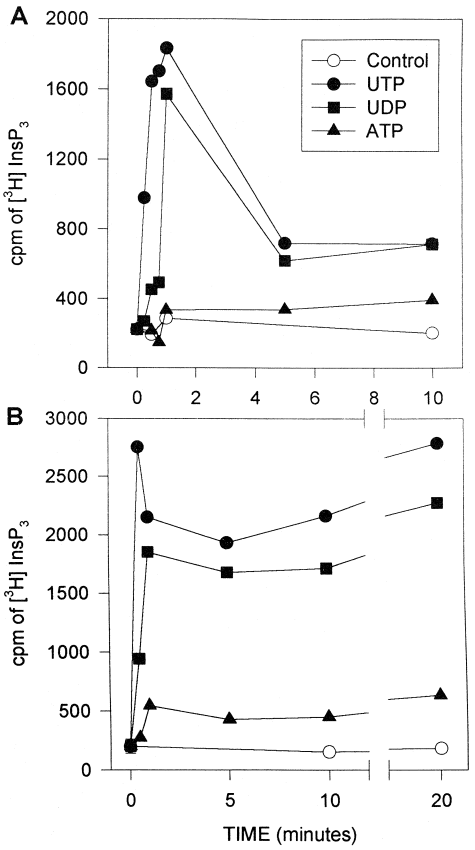

Fig. 1. Time course of $InsP_3$ accumulation in 1321N1 cells expressing the human $P2Y_4$ receptor. [^{3}H]inositol-labeled cells were incubated for the indicated time with UTP (100 μM), UDP (100 μM) and ATP (100 μM) in the absence of 10 mM LiCl (panel A) or in its presence (panel B). The data represent the mean of triplicate points and are representative of two independent experiments.

UTP, both at 30 s and 20 min. At 30 s, the response to 10 μM UTP was fully antagonized by 2 mM ATP (Fig. 2A), corresponding to the fact that ATP has no effect on the human $P2Y_4$ receptor at this early time. At 20 min, an inhibition of 62 ± 11% of the UTP effect (10 μM), corresponding to the difference between the UTP and the ATP effects, was observed in the presence of 2 mM ATP (mean ± S.D. of five independent experiments) (Fig. 2B,C). The ATP concentration-inhibition curves were shifted to the right when the UTP concentration was increased, indicating the competitive nature of this inhibitory effect (Fig. 2A,B). On the other hand, at lower concentrations (30–300 μM), ATP enhanced the response to UTP by 29% (range 12–47%, mean of four experiments) (Fig. 2B,C). ADP, which had almost no effect per se (Communi et al., 1995a) and did not inhibit the action of UTP, reproduced that enhancement: in the presence of ADP (100 μM), the stimulation by UTP (10 μM) represented 158 ± 15% (mean of three independent experiments) of that by UTP alone (data not shown). However, this potentiating effect of ATP and ADP was not specific: indeed the action of carbachol mediated by muscarinic receptors endogenously expressed in the 1321N1 cells (Filtz et al., 1994) was also increased in the presence of these nucleotides. This observation was reproduced with cells transfected with the recombinant

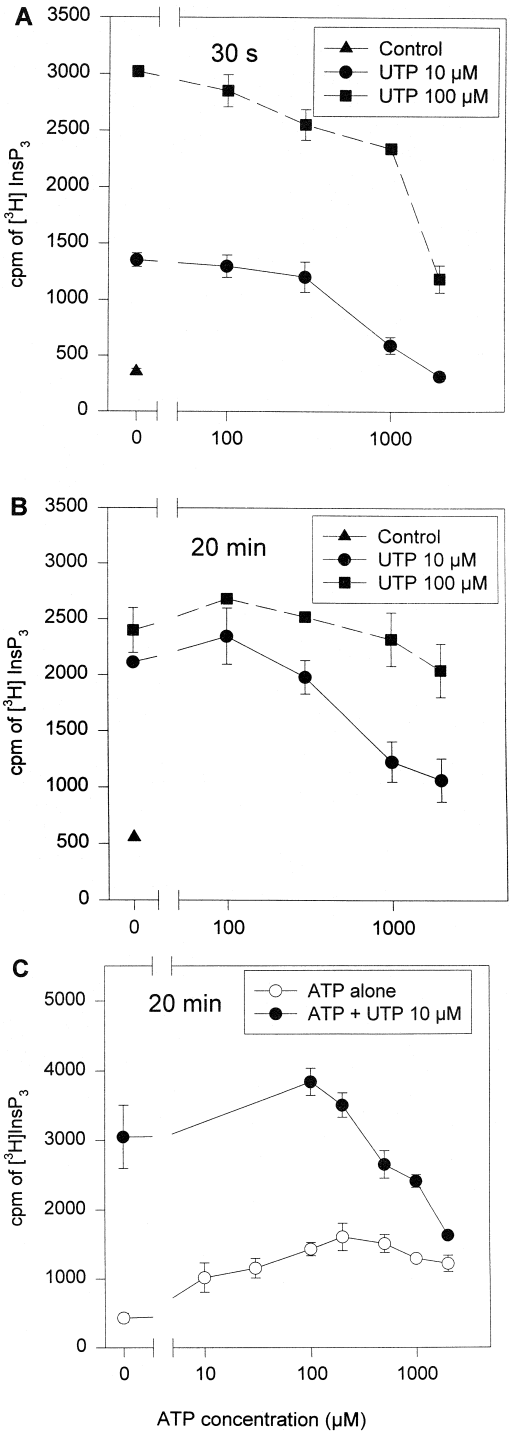

Fig. 2. Effect of ATP on the accumulation of $InsP_3$ induced by UTP in 1321N1-transfected cells. Concentration-action curves of ATP in the presence of UTP 10 or 100 μM at 30 s (panel A) and 20 min (panel B). Concentration-action curve of ATP with or without UTP (10 μM) at 20 min (panel C). The data represent the mean ± S.D. of triplicate points and are representative of two (panel A), five (panel B) or three (panel C) independent experiments.

$P2Y_4$-pcDNA3 plasmid or with the vector alone and was also obtained with AMP and adenosine (data not shown).

Nguyen et al. (1995) have reported that UTP was 100-fold more potent than UDP on $[Ca^{2+}]_i$, whereas in our initial experiments UTP and UDP were equipotent (Communi et al., 1995a). To resolve this issue, we compared the concentration-action curves of UTP and UDP on the $InsP_3$ production for several clones of transfected cells. The study was made at two times (Fig. 3): 30 s, a time close to that used by Nguyen et al. (1995) for their Ca^{2+} measurements, and 20 min, the time we used for the preliminary characterization of the $P2Y_4$ receptor (Communi et al., 1995a). In the new set of experiments performed on clone 11 (clone of 1321N1 transfected cells chosen for the pharmacological characterization), UTP appeared to be 10-fold more potent than UDP after a 20-min incubation and this difference was reproduced with two other clones (Fig. 3). The EC_{50} values for UTP and UDP were, respectively, 0.3 ± 0.1 and 3.3 ± 0.6 μM in clone 2, 2.4 ± 0.1 and 19.8 ± 4.8 μM in clone 11 and 0.3 ± 0.1 and 3.2 ± 0.8 μM in clone 21 (mean ± S.D. of two independent experiments). At 30 s of incubation, it was not possible to determine EC_{50} values because the curves were clearly shifted to the right, but we can observe that the difference of potency between the two agonists was even more

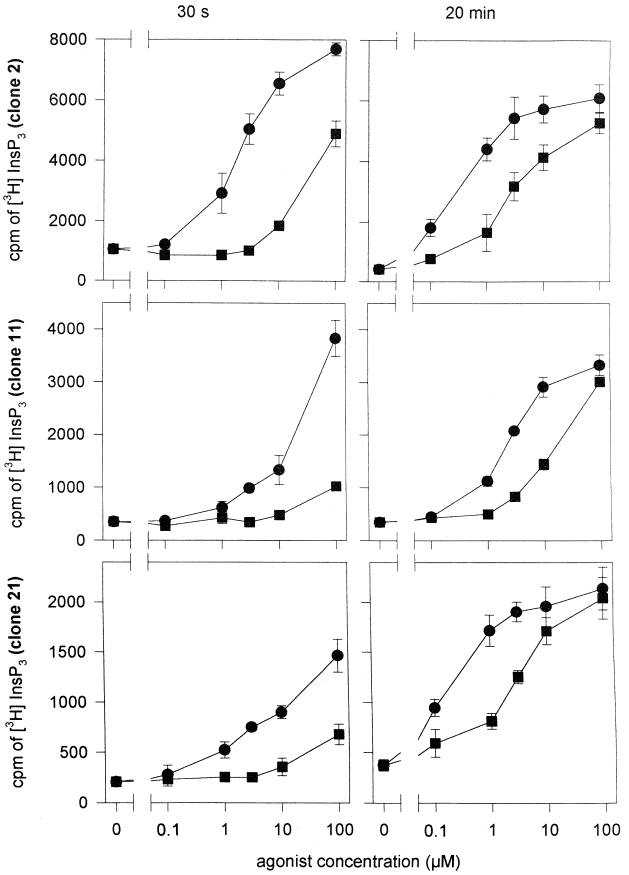

Fig. 3. Concentration-action curves of UTP and UDP on the $InsP_3$ accumulation in three different clones of 1321N1-transfected cells. The cells were incubated in the presence of various UTP (●) and UDP (■) concentrations (0, 0.1, 1, 3, 10 and 100 μM) for 30 s or 20 min. The data represent the mean ± S.D. of triplicate experimental points obtained in one representative experiment.

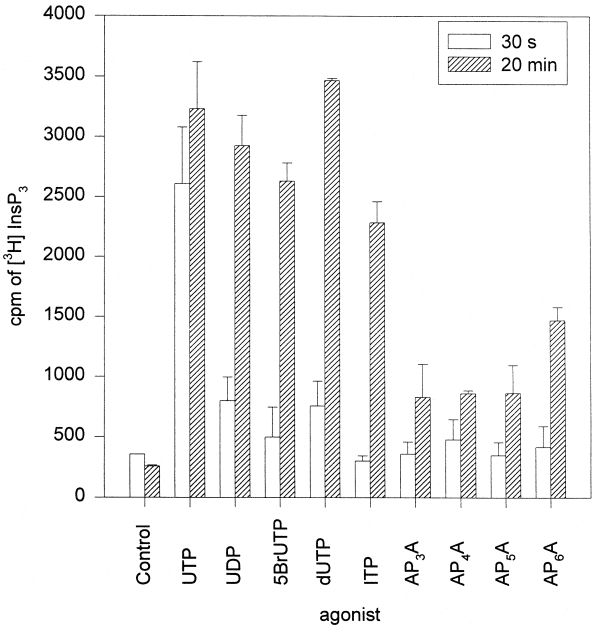

Fig. 4. Effect of various nucleotides on the $InsP_3$ production in 1321N1-transfected cells. The cells were incubated with UTP, UDP, 5BrUTP, dUTP, ITP, AP_3A, AP_4A, AP_5A and AP_6A at the same concentration of 100 μM or without agonist (control) for 30 s or 20 min. The data represent the mean ± S.D. of triplicate points and are representative of three independent experiments.

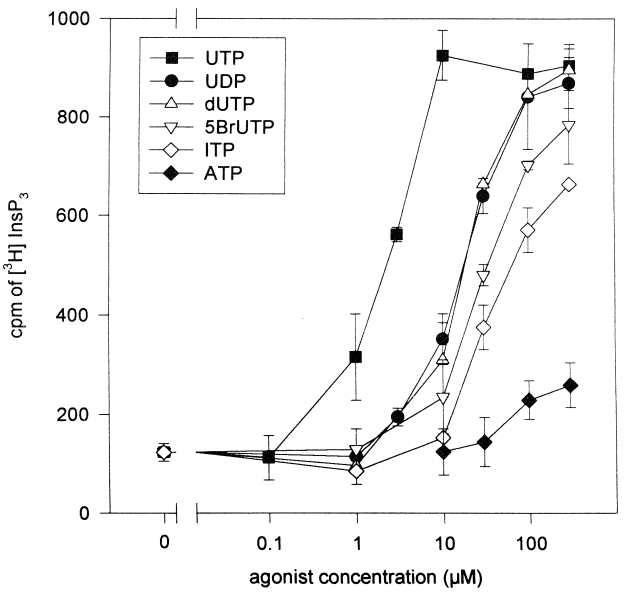

Fig. 5. Concentration-action curves of various nucleotides on the $InsP_3$ accumulation in 1321N1 cells expressing the human $P2Y_4$ receptor. 1321N1 cells were incubated in the presence of various concentrations of UTP, UDP, dUTP, 5BrUTP, ITP and ATP for a period of time of 20 min. The data are the mean ± range of duplicate experimental points obtained in an experiment representative of two.

striking (Fig. 3). Several clones, including clones 2, 11 and 21, were tested in binding studies with [α-^{32}P]UTP but no increase in specific binding was observed as compared to the cells transfected with the vector alone (data not shown).

In view of the time differences observed in Fig. 3, the testing of a range of nucleotides was performed at two times: 30 s and 20 min. As Fig. 4 shows, several agonists were barely or not active at 30 s (UDP, 5BrUTP, dUTP, ITP) whereas they produced a significant effect at 20 min. Full concentration-action curves were obtained at 20 min. The rank order of potency was: UTP > UDP = dUTP > 5BrUTP > ITP > ATP (Fig. 5). The EC_{50} values obtained were the following: EC_{50} UTP = 2.5 ± 0.6 μM, EC_{50} UDP = 19.5 ± 3.9 μM (mean ± S.D. of eight independent experiments), EC_{50} dUTP = 20.0 ± 2.3 μM, EC_{50} 5BrUTP = 27.1 ± 1.9 μM and EC_{50} ITP = 32.8 ± 5.4 μM (mean ± S.D. of two independent experiments). The approximate EC_{50} value obtained for ATP was: 43 ± 12 μM (mean ± S.D. of five independent experiments). The diadenosine polyphosphates also increased the $InsP_3$ production in transfected cells with EC_{50} between 3 and 7 μM (data not shown), but their maximal effect was only 20–25% of that of UTP, a value close to that of ATP (range of four independent experiments) (Fig. 4). UMP, uridine, AMP, adenosine and ATPγS were without any effect (data not shown).

No specific antagonist is available for any P2Y subtype. Nonetheless, several non-selective antagonists, such as suramin, reactive blue 2 or PPADS have been tested on P_2 receptors and their relative actions on these subtypes may constitute a mean to discriminate them (Boyer et al., 1994). We, therefore, tested the ability of these three antagonists to inhibit the UTP response in the model of the human $P2Y_4$ receptor. As we can see on Fig. 6, PPADS appeared to be the most active antagonist (73 ± 14% inhibition; IC_{50} around 15 μM (data not shown)), suramin was

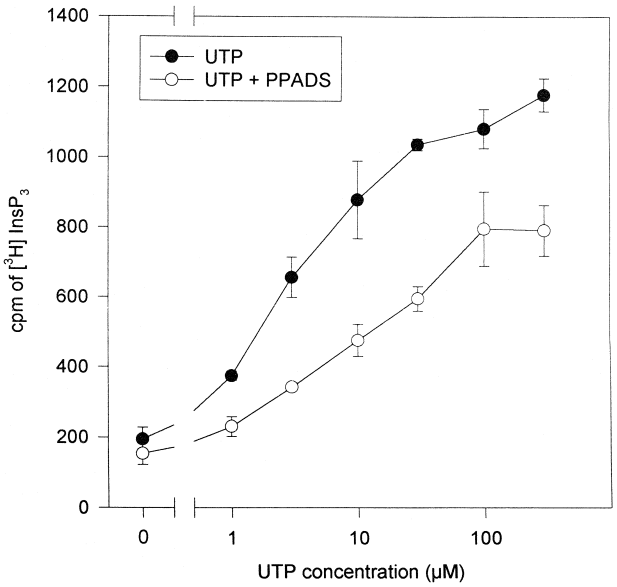

Fig. 7. Effect of PPADS on the UTP stimulation of $InsP_3$ in 1321N1-transfected cells. The cells were exposed to various concentrations of UTP in the presence or in the absence of PPADS (100 μM) for 20 min. The data are the mean ± S.D. of triplicate points obtained in an experiment representative of two.

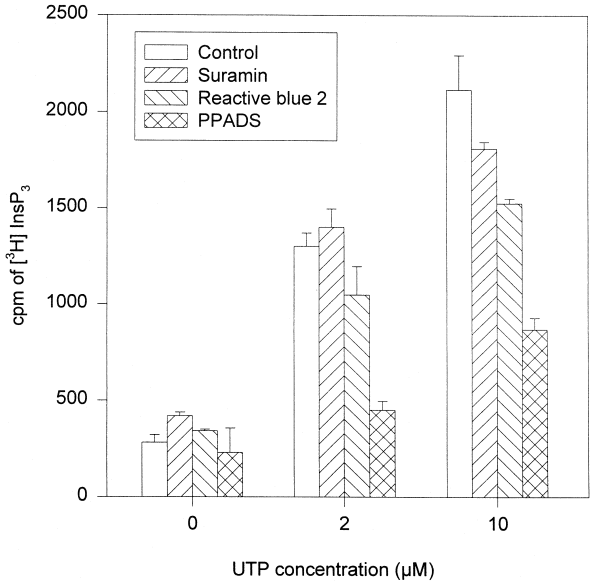

Fig. 6. Action of various P_2 antagonists on the $InsP_3$ production induced by UTP in 1321N1-transfected cells. Cells were incubated in the presence of suramin, reactive blue 2 and PPADS at a concentration of 100 μM and different UTP concentrations (0, 2 and 10 μM) for 20 min. The data represent the mean ± S.D. of triplicate points and are representative of two independent experiments.

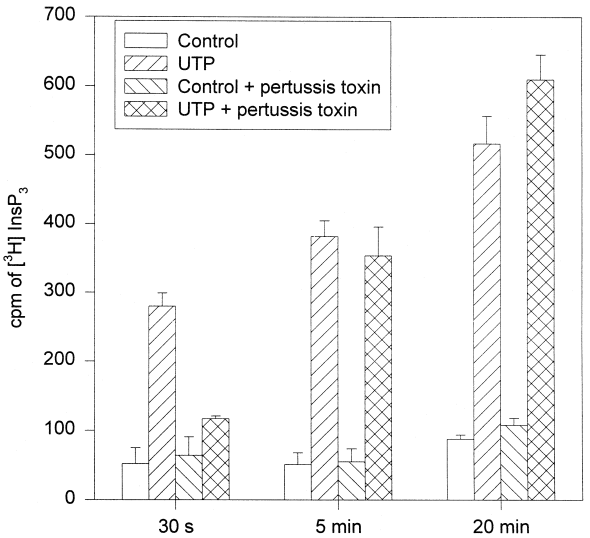

Fig. 8. Effect of pertussis toxin on the UTP-induced accumulation of $InsP_3$ in 1321N1 cells expressing the human $P2Y_4$ receptor. The cells were pre-incubated for 18 h in the presence or in the absence of 20 ng/ml pertussis toxin. The cells were then incubated with or without UTP 100 μM and with or without pertussis toxin (20 ng/ml) for various times: 30 s, 5 min or 20 min. The data represent the mean ± S.D. of triplicate points and are representative of two independent experiments.

inactive, and reactive blue 2 produced an inhibition of 33 ± 5% of the UTP response (mean ± S.D. of two independent experiments). Fig. 7 shows the mixed nature of the antagonism by PPADS of the UTP response: it affects both the EC_{50} value and the maximal effect of UTP. The EC_{50} value for UTP in the absence of PPADS was 3.3 ± 0.6 and 12.2 ± 4.5 μM in the presence of 100 μM PPADS (mean ± S.D. of two independent experiments).

The effect of pertussis toxin (20 ng/ml, 18 h pre-treatment) was studied at different times after UTP (100 μM) addition (Fig. 8). The UTP response was clearly inhibited at 30 s (62 ± 5% of inhibition: mean ± S.D. of two independent experiments), whereas no significant effect was observed at 5 and 20 min.

4. Discussion

We have characterized the time-course of the $InsP_3$ formation induced by UTP in 1321N1 cells stably expressing the human $P2Y_4$ receptor. The response to UTP was clearly biphasic with an early peak reached at 30 s which showed little sensitivity to LiCl, and a more sustained accumulation which was strongly amplified by LiCl. The H_1 and muscarinic receptors mediated responses, previously described in 1321N1 cells (Nakahata et al., 1986; Nakahata and Harden, 1987), have a clearly distinct time-course: early peak at 30 s followed by a return to the baseline within 1 min for the histamine response, rapid and sustained response with no evidence for desensitization for the muscarinic stimulation. ATP was able to induce an accumulation of $InsP_3$ in 1321N1 cells expressing the $P2Y_4$ receptor, but with a much slower time-course than UTP. No effect could be seen at 30 s explaining the lack of $[Ca^{2+}]_i$ rise in Nguyen et al. (1995)'s study. At later times, ATP stimulated the formation of $InsP_3$, but its maximal effect was less than that of UTP. Furthermore, ATP inhibited the stimulatory effect of UTP in a manner dependent on both ATP and UTP concentrations. Inhibition was total at 30 s and partial at 20 min. Thus, ATP was a pure antagonist at early times and behaved as a partial agonist at later times. The apparent contradiction between the data of Nguyen et al. (1995) showing that ATP is an antagonist of the $P2Y_4$ receptor and those of Communi et al. (1995a) indicating that it behaves as a partial agonist has, thus, been resolved. Even more striking was the time-dependent differential sensitivity to pertussis toxin inhibition: the early formation of $InsP_3$ in response to UTP was strongly inhibited by pertussis toxin, whereas this inhibition was no longer detectable at later times. This suggests the involvement of two distinct G-proteins in the coupling between the $P2Y_4$ receptor and phospholipase C: a G_i-protein at early times and a protein of the $G_{q/11}$ family later. The role of a G_i-protein is consistent with the presence of a threonine residue at the end of the third intracytoplasmic loop (Thr^{246}). Such a threonine residue is indeed a distinctive feature of G_i-coupled receptors (Liu et al., 1995), and its mutation leads to their constitutive activation (Lefkowitz et al., 1993; Samama et al., 1993; Ren et al., 1993). After 30 s, only UTP was able to produce a significant stimulation of $InsP_3$ accumulation at a concentration of 100 μM. In agreement with the calcium data of Nguyen et al. (1995), UTP was definitely more potent than UDP even at later times. The rank order of potency of a wider range of nucleotides could be determined after a longer incubation (20 min) and was as follows: UTP > UDP = dUTP > 5BrUTP > ITP > ATP. The significant though much smaller stimulatory effect of diadenosine polyphosphates is reminiscent of their action on recombinant $P2Y_2$ receptors, recently demonstrated in the same expression system (Lazarowski et al., 1995). At early times, the $P2Y_4$ receptor has, thus, a strong selectivity for UTP and couples to a G_i-protein, whereas at later times, it is activated by a wider range of agonists and is coupled to another G-protein. It is tempting to link these data and to suggest the hypothesis that the $P2Y_4$ receptor can assume two distinct activation states, which differ in terms of affinity for nucleotides and G-protein coupling. Evidence supporting such a concept has already been reported in the literature (Perez et al., 1996; Spengler et al., 1993), although it remains scarce. It has been shown, for instance, that the PACAP (pituitary adenylyl cyclase-activating peptide) is preferentially coupled to adenylyl cyclase when it is occupied by PACAP-27, whereas the binding of PACAP-38 induces a selective activation of phospholipase C (Spengler et al., 1993).

The $P2Y_4$ receptor had some selectivity for known P_2 receptors antagonists: PPADS was more active than reactive blue 2, while suramin was barely active. This profile is distinct from that observed with other receptors. PPADS, reactive blue 2 and suramin are all antagonists of the $P2X_1$, $P2X_2$ and $P2X_3$ receptors, whereas the $P2X_4$ subtype is insensitive to all of them (Valera et al., 1994; Brake et al., 1994; Chen et al., 1995; Bo et al., 1995). The rank order of potency is PPADS > suramin > reactive blue 2 at the phospholipase C-coupled $P2Y_1$ receptor of turkey erythrocyte and reactive blue 2 > suramin > > PPADS for the yet uncharacterized receptor of C6 glioma cells which is coupled to adenylyl cyclase inhibition (Boyer et al., 1994). PPADS is not an antagonist of the $P2Y_2$ receptor (Brown et al., 1995) and is less active than suramin on the $P2Y_6$ receptor (Chang et al., 1995): among the P_2 receptors, the $P2Y_4$ receptor is, thus, characterized by a unique profile of antagonist sensitivity.

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