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Human $P2Y_2$ receptor polymorphism: identification and pharmacological characterization of two allelic variants

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1 In the process of cloning the human $P2Y_2$ receptor in order to establish 1321N1 cell lines expressing this receptor, we detected a gene polymorphism characterized by an arginine 334 to cysteine 334 transition.

2 The frequency distribution of the polymorphism was studied in a European population. We observed that 66% of the tested persons are homozygotes R/R, 29% are heterozygotes R/C and 5% are homozygotes C/C. The frequency of the R allele was 0.8 versus 0.2 for the C allele.

3 We stably expressed each form of the human $P2Y_2$ receptor into 1321N1 cells and isolated clones by limiting dilution. The effects of nucleotides and antagonists on inositol trisphosphate accumulation and cyclic AMP formation were compared between the two cell lines.

4 The time-courses of inositol trisphosphate accumulation as well as concentration-response curves characterizing the effects of UTP, ATP, AP_4A and ATPγS were mostly similar, except for slight kinetic differences (slower time-course with the ^{334}C form).

5 The sensitivity to pertussis toxin of inositol trisphosphates accumulation was critically dependent on the agonist concentration and stimulation duration, suggesting the involvement of a $G_{i,0}$ protein during the early stimulation by low nucleotide concentrations. No inhibition of cyclic AMP accumulation could be detected. These properties were observed with both polymorphic receptors.

Keywords: Human $P2Y_2$ receptor; polymorphism; palmitoylation; pharmacological characterization; $G_{i,0}$ and $G_{q,11}$ coupling; antagonists; adenylyl cyclase modulation

Abbreviations: AP_4A, P^1,P^4-di(adenosine-5′) tetraphosphate; ATPγS, adenosine 5′-O(3-γ-thio)triphosphate; cAMP, cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; FK, forskolin; $InsP_3$, inositol trisphosphates; PCR, polymerase chain reaction; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; PTX, pertussis toxin; RB2, reactive blue 2; T_{50}, time for $InsP_3$ response to decline to 50% of its maximum; T_{95}, time to reach 95% of maximal $InsP_3$ response

Introduction

The nucleotidic receptors or P_2 receptors are subdivided in two classes: P2X receptors, which are ATP-gated cation channels, and P2Y receptors, which are heptahelical G-coupled receptors (Abbrachio & Burnstock, 1994). The P2Y family encompasses selective purinoceptors, the $P2Y_1$ (Webb *et al*., 1993) and $P2Y_{11}$ receptors (Communi *et al*., 1997), selective pyrimidinoceptors, the $P2Y_3$ (Webb *et al*., 1996a), $P2Y_4$ (Communi *et al*., 1995a), and $P2Y_6$ (Chang *et al*., 1995) receptors, and nucleotidic receptors responsive to both adenine and uracil nucleotides, such as the $P2Y_2$ (Lustig *et al*., 1993) and the $P2Y_8$ (Bogdanov *et al*., 1997) receptors. All these P2Y receptors are coupled to the phospholipase C pathway; moreover the recently cloned $P2Y1_{11}$ receptor is also coupled to adenylyl cyclase stimulation. The inclusion of other receptors, the $P2Y_5$ (Webb *et al*., 1996b; Li *et al*., 1997), $P2Y_9$ also called $P2Y_5$-like (Janssens *et al*., 1997) and $P2Y_{10}$ receptors (EMBL database: accession number AA747912) within the P2Y family remains controversial. As for the $P2Y_7$ receptor (Akbar *et al*., 1996), it has been recharacterized as the leukotriene B4 receptor (Yokomizo *et al*., 1997).

The $P2Y_2$ receptor, activated equipotently by UTP and ATP, is widely expressed among tissues and cell lines. In endothelial cells, the activation of the $P2Y_2$ receptor causes the release of prostacyclin (PGI_2) and nitric oxide (NO) which act as vasodilators and inhibitors of platelet aggregation (Boeynaems & Pearson, 1990). The $P2Y_2$ receptor is also expressed in airway epithelial cells where its activation increases chloride permeability, *via* the activation of Ca^{2+}-dependent ORCC channels (Outward Rectifying Chloride Channel) distinct from the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator). Therefore UTP and ATP sprays have been advocated as a therapy of cystic fibrosis (for review: Donaldson & Boucher, 1998). These roles of the $P2Y_2$ receptor make it a target for the discovery of new therapeutic agents. In the process of cloning the human $P2Y_2$ gene in view to establish stable recombinant 1321N1 cell lines, we identified two forms of the human $P2Y_2$ receptor differing by the substitution of C base for T at nucleotide 1000, leading to an arginine to cysteine substitution at position 334. In this paper, we have determined the frequency of the polymorphism and compared the pharmacological properties of the two forms of the $P2Y_2$ receptor.

Methods

Cloning of the human $P2Y_2$ receptor

The complete open reading frame of the receptor was amplified by polymerase chain reaction (PCR) using specific primers

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(Forward: atgggaattctggtcagggcgatggcagc; Reverse: atcatctagaagtgttctgctcctacagccgaatg). These primers were synthesized on basis of the 5′ and 3′ extremities of the coding sequence of the human $P2Y_2$ receptor (Parr *et al*., 1994). The amplified product was subcloned into the expression vector peFIN (Euroscreen) and sequenced using eight specific primers as templates for the sequencing reaction. These primers were chosen at different positions of the receptor allowing us to sequence the receptor on both strands. The technology used for sequencing was the ABI PRISM Dye terminator cycle sequencing Ready reaction kit (Perkin Elmer) on a Applied Biosystems 373 DNA sequencer.

Genotyping

One hundred genomic DNA samples randomly taken among a European population were used in PCR to amplify the complete $P2Y_2$ coding sequence. The 3′ carboxyterminal tail of the PCR product was sequenced using a specific primer located just after the seventh transmembrane region as template. We determined a sequence of approximately 200 bases of the $P2Y_2$ gene, which includes the codon of the amino acid 334.

Cell culture and transfection

The coding sequence of each form was inserted into the expression vector peFIN. Astrocytoma 1321N1 cells were stably transfected with the recombinant peFIN plasmid using the calcium phosphate precipitation method as previously described (Velu *et al*., 1989). The wild-type cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FCS, 100 U ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, 2.5 $\mu g\ ml^{-1}$ amphotericin B and 1% of sodium pyruvate. The transfected cells were harvested in the same medium supplemented by 400 $\mu g\ ml^{-1}$ G418. From the pool of transfected 1321N1 cells, individual clones were isolated by limiting dilution with the purpose of selecting clones with high expression of the receptor.

Inositol phosphates measurements

150,000 transfected 1321N1 cells were labelled for 24 h with 5 $\mu Ci\ ml^{-1}$ myo-D-2[^{3}H]-inositol in inositol free DMEM containing 5% FCS, antibiotics, amphotericin B and sodium pyruvate. The cells were stimulated with the agonist without changing the labelling medium and washing the cells. This procedure avoids the release of intracellular nucleotides by 1321N1 cells, that occurs easily when these cells are mechanically disturbed (Lazarowski *et al*., 1995). Incubations were terminated by the addition of ice cold 3% perchloric acid solution. Extraction and isolation of inositol trisphosphates ($InsP_3$) on Dowex AG1-X8 columns were performed as described (Communi *et al*., 1995b). All assays were performed at least twice with triplicate samples. The data are expressed either as percentage of the maximal $InsP_3$ formation, which did not differ by more than 25% between the tested cell clones, or in c.p.m. EC_{50} values were determined using SigmaPlot v2.0 Curve Fit. Student's *t*-tests were performed with SigmaPlot v2.0 software.

Cyclic AMP measurements

Stably transfected 1321N1 cells were seeded on Petri dishes (150,000 cells) in complete DMEM medium and cultured overnight. The cells were first preincubated 30 min with 25 μM rolipram, a phosphodiesterase inhibitor, and then incubated for 10 min with forskolin (FK) at 1 μM, in the presence of rolipram 25 μM. Finally, they were stimulated for various times (2–15 min) by nucleotides, in a medium supplemented with rolipram and FK. The incubation was stopped by the addition of HCl 0.1 M. After evaporation to dryness, the samples were diluted in water as required. Cyclic AMP was quantified by radioimmunoassay after acetylation (Brooker *et al*., 1979). Assays were performed at least three times with triplicate samples.

Results

Cloning of the human $P2Y_2$ receptor

In order to amplify the human $P2Y_2$ receptor, PCR were performed on human genomic DNA at high stringency using specific primers, synthesized on basis of the 5′ and 3′ extremities of the published coding sequence of the human $P2Y_2$ receptor (Parr *et al*., 1994). The amplified open reading frame was subcloned into the peFIN vector and sequenced to detect mutated products generated by the PCR. Twelve amplified fragments derived from four separate PCR (using each time a new DNA sample) were totally sequenced on both strands. A comparison of the obtained sequences to the previously published sequence revealed two differences. In all the amplified fragments, an adenine was present instead of a guanine at position 1049 of the nucleotidic sequence, resulting in the replacement of a glycine by a glutamic acid at position 350 of the amino acid sequence ($^{350}G \rightarrow {}^{350}E$). This difference was later confirmed in the 100 samples of genomic DNA which have been analysed (see below) and indicates a sequencing error in the original sequence. The second difference involves the replacement of a cytosine by a thymine at position 1000, leading to the substitution of arginine 334 by a cysteine ($^{334}R \rightarrow {}^{334}C$). As this difference was observed only in some clones, it was likely to correspond to a polymorphism of the $P2Y_2$ receptor gene (Figure 1).

Frequency of the polymorphism

In order to define the frequency of the polymorphism, we amplified the complete human $P2Y_2$ coding sequence from 100 human genomic DNA samples randomly taken among a European population. The PCR products were sequenced using a primer recognising a region of the gene located just before the region of interest (after the seventh transmembrane region). We determined for each DNA a partial sequence of approximately 200 bases, which includes the amino acid present at position 334. This method allowed us to confirm unambiguously the presence of a polymorphism and also the presence of ^{350}E instead of ^{350}G. Moreover, we determined the frequencies of the different genotypes: R/R = 66%, R/C = 29%, and C/C = 5%; R = 0.8 and C = 0.2.

Pharmacological characterization of the two human $P2Y_2$ variants: time-course and concentration-response curves

We first studied the time-course of $InsP_3$ accumulation induced by UTP, ATP or ATPγS at 100 μM in the human $P2Y_2$ ^{334}R or ^{334}C clones, in absence of LiCl. As can be seen in Figure 2, there was a rapid rise during the first 15 s, a phase of slower accumulation and a plateau followed by a decrease of the intracellular $InsP_3$ level. A comparison between the kinetic

curves of the two receptors revealed slight but reproducible differences. With the ^{334}R variant, the time-course was characterized by a faster accumulation of intracellular $InsP_3$: the time to reach 95% of the maximal response (T_{95}), determined by graphic interpolation, was about 15 min for the ^{334}R variant as compared to 30 min for the ^{334}C variant (Table 1). When ATPγS (100 μM) was the agonist, both the onset of the response and its decay were more rapid with the ^{334}R form as compared to the ^{334}C variant (Figure 2; Table 1).

Full concentration-response curves were obtained after a 30 s incubation with the most potent nucleotides (UTP, ATP, AP_4A and ATPγS) (Lazarowski *et al*., 1995). For both variants, the order of potency of the agonists was conserved (UTP > ATP > > AP_4A > ATPγS) and the EC_{50} obtained were almost identical (Figure 3). We also tested the effects of other molecules such as UDP, UDP-glucose, ADP, GTP, GDP, AP_3A, AP_5A and NAD^+ at 10 μM. GTP generated an almost maximal effect on the $InsP_3$ accumulation when compared to

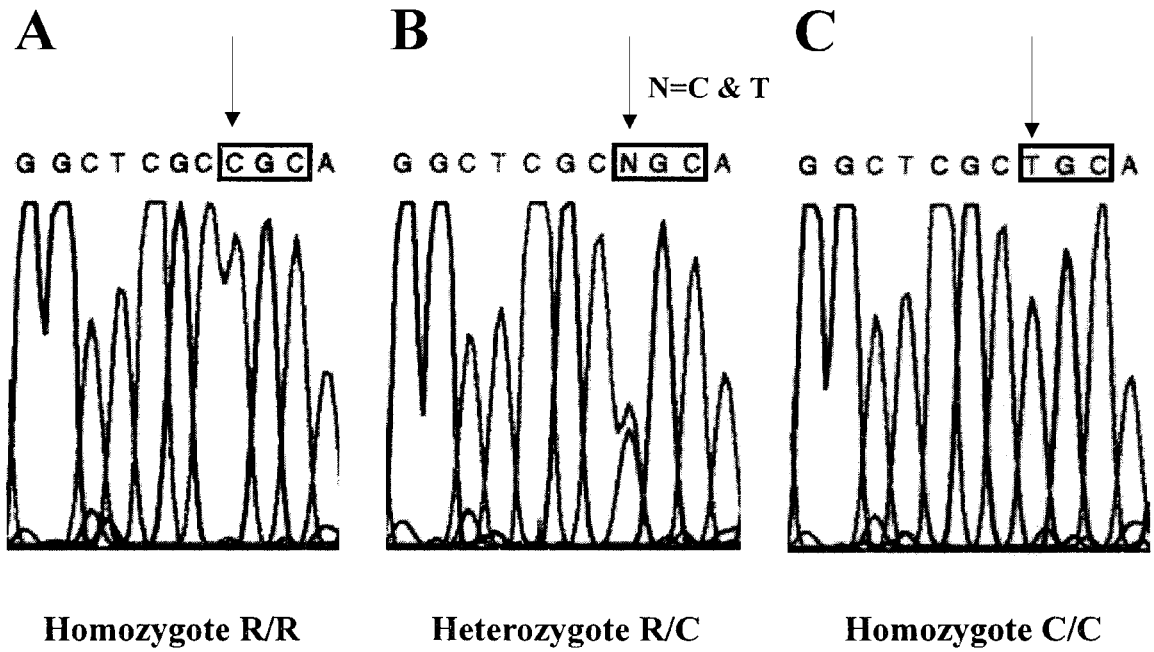

Figure 1 Comparison of the nucleotidic sequences of the two human $P2Y_2$ alleles. The sequencing was performed on three different genomic DNA samples representative of a homozygote R/R (A), a heterozygote R/C (B) and a homozygote C/C (C). The nucleotide 1000 of the coding sequence responsive for the polymorphism is indicated by an arrow. The codon of the amino acid 334 modified by the mutation is represented in a frame.

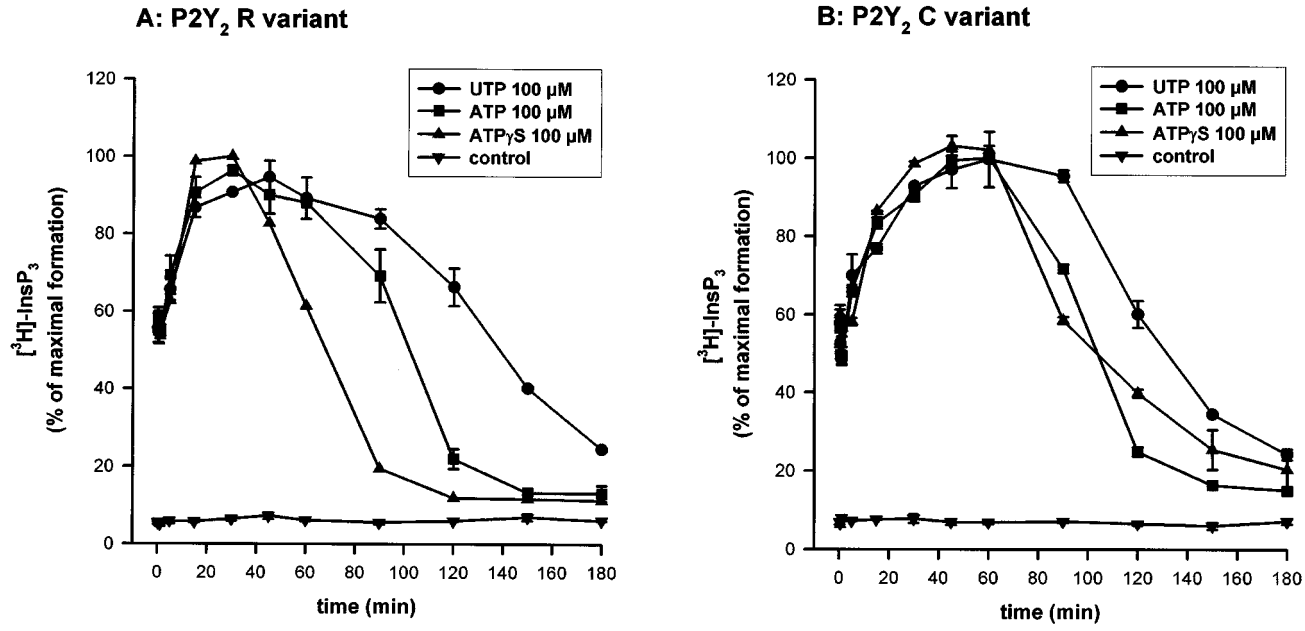

Figure 2 Time-course of $InsP_3$ accumulation in 1321N1 cells expressing the human $P2Y_2$ receptor R variant (A) and C variant (B). ^{3}H-inositol labelled cells were incubated for the indicated time with UTP, ATP or ATPγS (100 μM) in absence of LiCl. The data represent the mean $\pm$ s.d. of triplicate points and are representative of three independent experiments. 100% of $InsP_3$ formation correspond to 1832 c.p.m. for the ^{334}R variant and 1634 c.p.m. for the ^{334}C variant.

Table 1 Quantitative comparison of the kinetics of nucleotide-induced $InsP_3$ accumulation between the two $P2Y_2$ variants

Agonists	T_{95} ^{334}R (min)	T_{95} ^{334}C (min)	P
UTP	14.8±0.7	29.4±0.7	0.001
ATP	17.4±1.0	36.5±1.5	0.001
ATPγS	13.6±0.2	29.3±0.8	<0.001
	T_{50} ^{334}R (min)	T_{50} ^{334}C (min)	
UTP	148.1±1.9	141.0±1.9	0.098
ATP	110.6±1.1	109.0±0.2	0.304
ATPγS	73.5±0.2	113.4±0.5	<0.001

T_{95} and T_{50} are respectively the mean time for reaching 95% of the maximal $InsP_3$ formation and the mean time for $InsP_3$ response to decline to 50% of its maximum. Data (mean± s.e.mean) are representative of the three independent experiments described in Figure 2. *P* is the level of significance of the difference between the ^{334}R and ^{334}C variants, obtained by Student's *t*-test.

the UTP response (80% of the UTP response), whereas AP_3A increased only slightly the $InsP_3$ level (30% of the UTP response). This last experiment did not reveal differences between the two polymorphic receptors (data not shown).

Study of the effects of the antagonists

The inhibitory action of suramin, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and reactive blue 2 (RB2) on the stimulation of phospholipase C induced by UTP was investigated in the $P2Y_2$ ^{334}R or ^{334}C 1321N1 transfected cells. These cells were preincubated 30 s with the antagonists at a concentration of 100 μM before the addition of UTP at various concentrations (30, 100 and 300 nM) for 30 s. As represented in Figure 4, the rank order of inhibition was RB2 > > suramin > PPADS. Exactly the same order and degree of inhibition were observed for both variants (data not shown).

Study of the $P2Y_2$ coupling to the $G_{i,0}$ protein

The effect of pertussis toxin (PTX) (24 h pretreatment at 100 ng ml^{-1}) was tested on the action of UTP and ATPγS at different concentrations (10 nM – 100 μM) and for different periods of stimulation (30 s and 15 min). After 30 s of stimulation, PTX produced a parallel shift in concentration-response curves with no change in maximum. After a 15 min stimulation, the concentration-response curves were shifted to higher concentrations, both for UTP and ATPγS, and the PTX effect was no longer detectable (Figure 5). The two variants had the same sensitivity to PTX (data not shown).

Study of the modulation of the adenylyl cyclase pathway

The transfected cells, preincubated with forskolin (FK) 1 μM, were stimulated with increasing concentrations of UTP (10 nM – 10 μM) for 15 min. We observed a slow increase of the level of intracellular cyclic AMP leading to a 30% augmentation when stimulated by UTP as compared to the control level (Figure 6A). The $P2Y_2$ expressing cells were also stimulated for various time (2 – 15 min) by low UTP concentrations (10, 30 and 100 nM), in the presence of FK 1 μM. No inhibition of the cyclic AMP accumulation could be observed (Figure 6B). The effect of nucleotides (i.e. UTP, ATP and AP_4A at 10 μM) was also tested in absence

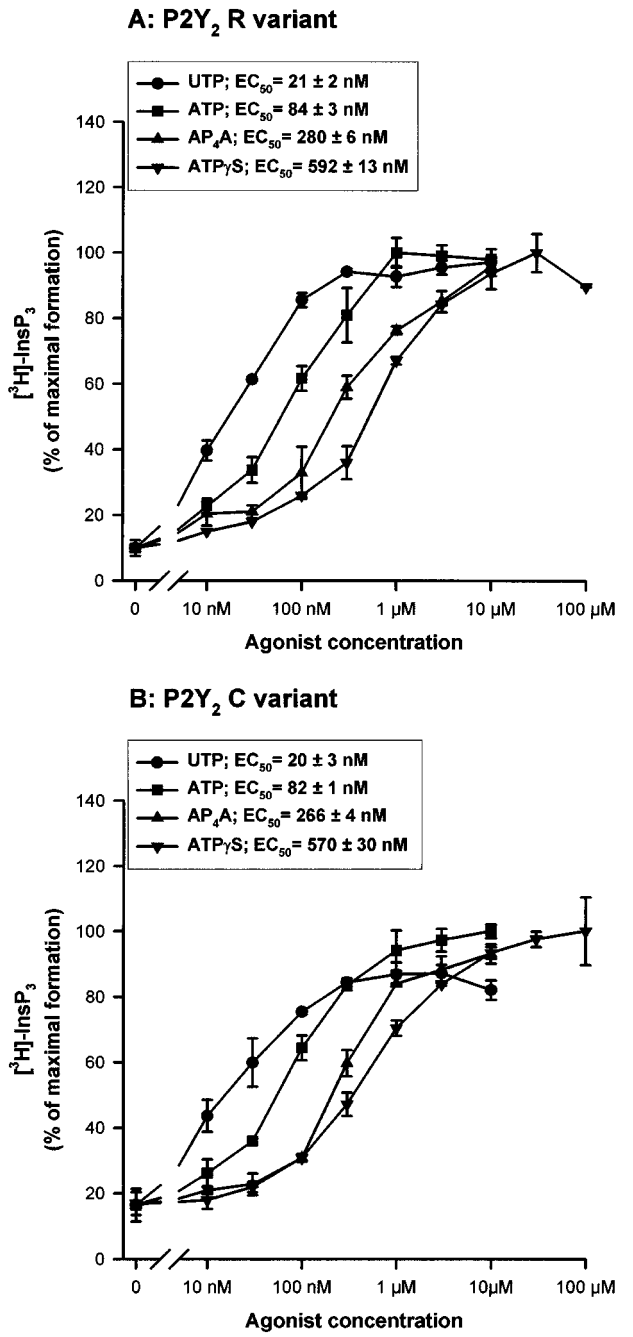

Figure 3 Concentration-response curves of various nucleotides on the $InsP_3$ accumulation in 1321N1 cells expressing the human $P2Y_2$ receptor R variant (A) and C variant (B). The cells, labelled overnight with ^{3}H-inositol, were incubated in presence of various concentrations of UTP, ATP, AP_4A and ATPγS for 30 s. The data are the mean±s.d. of triplicate experimental points and are representative of two separate experiments. The EC_{50} values (means ±range) shown in the inset were calculated on basis of the two experiments. 100% of $InsP_3$ formation correspond to 1644 c.p.m. for the ^{334}R variant and 1512 c.p.m. for the ^{334}C variant.

of FK. UTP generated a 2 fold increase of the cyclic AMP level after a 15 min incubation. AP_4A and especially ATP displayed a much stronger stimulation. A significant part of this response is likely to be due to the degradation of adenine nucleotides into adenosine acting on A_2 receptors (Communi *et al*., 1997). These experiments did not reveal any significant difference between the two polymorphic receptors (data not shown).

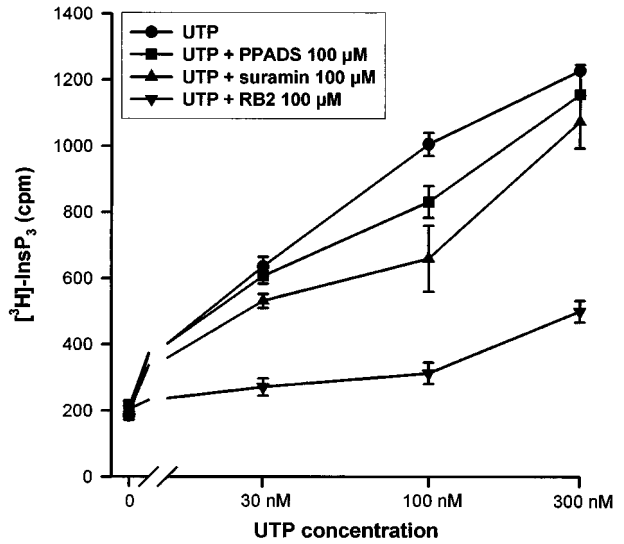

Figure 4 Effect of suramin, PPADS and RB2 (100 μM) in the $InsP_3$ formation generated by the stimulation of the human $P2Y_2$ R variant (closely similar results were observed for the $P2Y_2$ C variant) with UTP. The ^{3}H-inositol labelled cells were preincubated 30 s with the antagonist, before the addition of UTP (30, 100 or 300 nM) for another 30 s. The data represent the mean $\pm$ s.d. of triplicate points and are representative of two independent experiments.

Discussion

The P_2 receptors are known to be activated by extracellular nucleotides and to mediate many physiological and pharmacological responses in different cells and organs (Dubyak & El-Moatassim, 1993). The $P2Y_2$ receptor, which is activated by UTP, ATP and also AP_4A (Lazarowski *et al*., 1995), belongs to that family. Recently, the human $P2Y_2$ receptor, cloned from the human airway epithelial cell line CF/T43 (Parr *et al*., 1994), has acquired an increasing importance because of its role in the treatment of respiratory diseases such as cystic fibrosis (Donaldson & Boucher, 1998).

In the process of cloning the human $P2Y_2$ receptor coding sequence in order to establish a stable recombinant astrocytoma 1321N1 cell line as a tool for drug screening, we identified two forms of the $P2Y_2$ receptor. One corresponded to the sequence published by Parr *et al*. (1994), except for a single base difference (1049G – 1049A) leading to the replacement of glycine 350 by glutamic acid: as the difference was found in all the DNA analysed, it has to correspond to a sequencing error in the published sequence. The second was characterized by the replacement of arginine 334 into a cysteine and was found only in a minority of the samples analysed indicating the existence of a gene polymorphism. Sequence analysis performed on 100 European genomic DNA samples randomly taken showed that 66% of individuals are homozygotes R/R, 29% are heterozygotes R/C and 5% are homozygotes C/C. These data are in complete agreement with the Hardy-Weinberg equilibrium ($R^2 + C^2 + 2RC = 1$; R^2 = homozygotes R/R frequency, C^2 = homozygotes C/C frequency and 2RC = heterozygotes R/C; R = 0.8 and C = 0.2). The experimental result fits the theoretical equilibrium, indicating that the two alleles of the $P2Y_2$ gene are segregated in a Mendelian manner.

The cysteine 334 located in the carboxy terminal tail close to the seventh transmembrane region constitutes a potential site of palmitoylation, a process known to modulate G-coupled receptors function (Mumby, 1997). This possibility reinforced

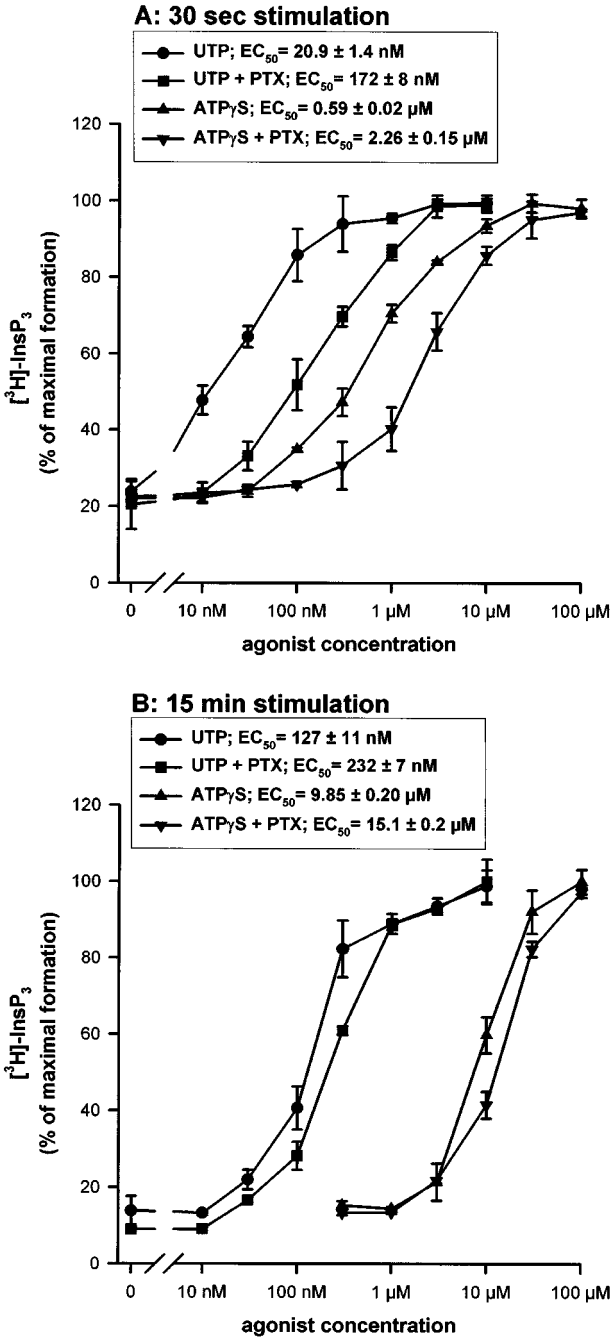

Figure 5 Effect of pertussis toxin on the UTP or ATPγS-induced accumulation of $InsP_3$ in 1321N1 cells expressing the human $P2Y_2$ C variant (similar results were obtained with the R variant). The cells were preincubated or not overnight in the presence of 100 ng ml^{-1} of pertussis toxin (PTX) and were then incubated in the presence of various concentrations of UTP or ATPγS for 30 s (A) or 15 min (B). The data represent the mean $\pm$ s.d. of triplicate points and are representative of three independent experiments. The EC_{50} values (means $\pm$ s.e.mean) shown in the inset were calculated on basis of the three experiments.

our interest in comparing the pharmacological properties of the two $P2Y_2$ receptors. Both variants were separately transfected into 1321N1 cells which were cloned by limiting dilution. Expression was documented by Northern blotting (data not shown) and functional assays. Time-course and concentration-response experiments were performed. The $InsP_3$ accumulation induced by UTP, ATP or ATPγS stimulation, in the absence of LiCl, was characterized by a

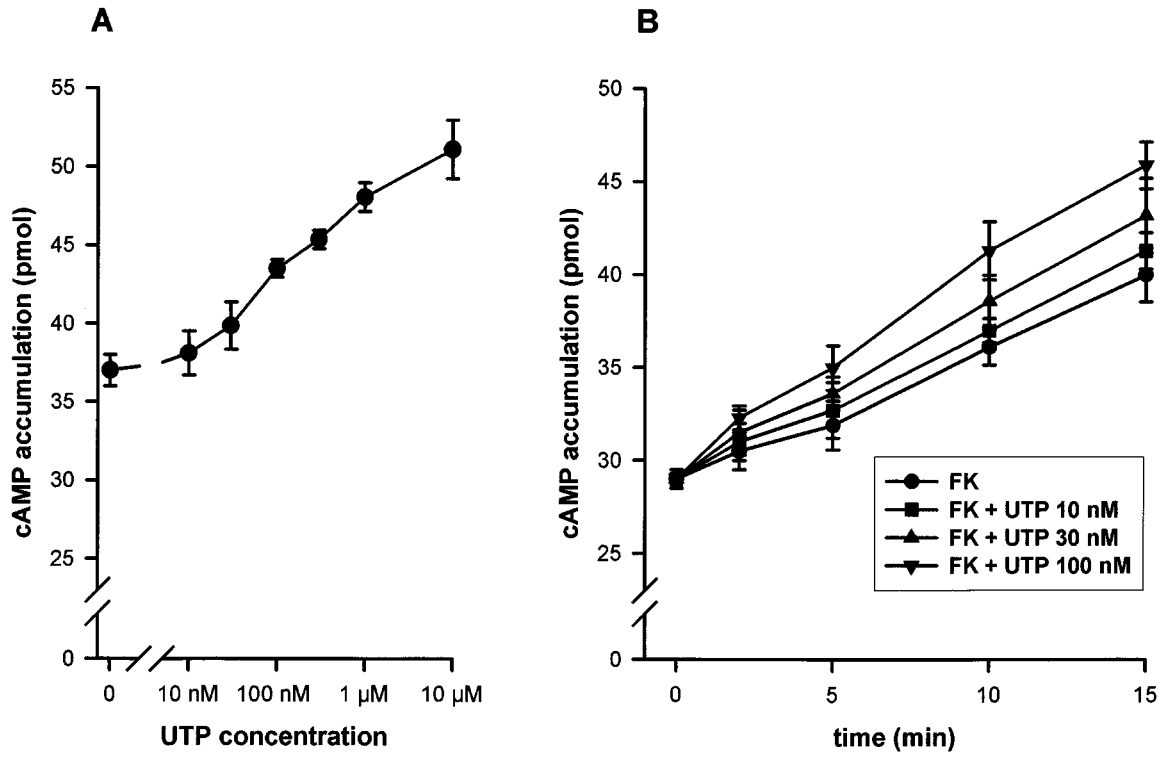

Figure 6 (A) Concentration-response curve of the UTP effect on cyclic AMP accumulation in 1321N1 cells expressing the human $P2Y_2$ R variant (similar results were observed for the C variant). The $P2Y_2$ expressing 1321N1 cells were stimulated 15 min with increasing concentrations of UTP. (B) Time-course of the UTP effect on cyclic AMP accumulation. 1321N1 cells expressing the R variant were stimulated by low concentrations of UTP (10, 30 or 100 mM) for various periods (2, 5, 10 or 15 min). For both experiments, prior to stimulation, the cells were preincubated 30 min with rolipram 25 μM, then 10 min with FK 1 μM and rolipram 25 μM before the addition of UTP, supplemented by FK and rolipram. The data represent the mean $\pm$ s.d. of triplicate points and are representative of three independent experiments.

very fast onset, followed by a slower increase, reaching a plateau before decreasing to the basal level. Interestingly, despite its resistance to hydrolytic enzymes, the decay of the ATPγS response was more rapid than that of a comparable concentration of UTP. The second phase of $InsP_3$ accumulation in response to nucleotides was slightly slower with the ^{334}C variant as compared to the ^{334}R form. The two variants were more clearly discriminated using ATPγS, a non-degradable agonist: with the ^{334}R form, the $InsP_3$ response had a more rapid onset and was also more transient, as compared to the ^{334}C variant. The concentration-response curves characterizing the stimulation by UTP, ATP, AP_4A and ATPγS were similar for both receptors and the EC_{50} were almost identical. We also studied the effects of antagonists of P2Y receptors (suramin, PPADS and RB2) on the $InsP_3$ formation. Under the experimental conditions used (30 s preincubation with the antagonist before the nucleotide addition), our results showed that suramin and PPADS were weak antagonists as previously described (Charlton *et al.*, 1996). Interestingly, we found that RB2 was the most active inhibitor of the $P2Y_2$ receptor. Obviously that rank order of potency might have been affected by the short preincubation time, since some of these agents are characterized by a slow equilibration (Leff *et al.*, 1990). These experiments did not reveal new differences between the two variants.

Moreover, in this work, we investigated the sensitivity to PTX and the interaction of the human $P2Y_2$ receptor with the adenylyl cyclase pathway. These experiments did not reveal additional differences between the two forms of receptor, but they shed new light on the coupling of the human $P2Y_2$ receptor to G proteins. In previous studies, the PTX sensitivity of the recombinant human $P2Y_2$ receptor was studied only at maximal agonist concentrations and shown to be partial: about 25% in the human $P2Y_2$ transfected 1321N1 cells (Parr *et al.*, 1994) and 35% in the mouse $P2Y_2$ transfected K-562 cells (Erb *et al.*, 1993). The inhibitory effect of PTX on the responses mediated by natively expressed $P2Y_2$ receptors was variable: partial in HL-60 cells (Dubyak *et al.*, 1988) and complete in BAEC (bovine aortic endothelial cells) (Motte *et al.*, 1993). In this study, we showed that the PTX sensitivity of the $P2Y_2$ receptor is critically dependent on the agonist concentration: the inhibition was very strong or complete at low nucleotide concentration but progressively disappeared at concentrations producing a maximal effect. We also showed that PTX sensitivity was greater at early times during the stimulation and disappeared at later times in parallel with a shift of the concentration-response curves to higher agonist concentrations. This shift is unlikely to be due to nucleotide degradation, since it was of the same magnitude for UTP and ATPγS. It might be due to a reduction in the number of receptors present on the surface of the cell or of the available pool of G protein able to couple to the receptor. These experiments suggest that at least two G-proteins are involved. At low concentrations of nucleotides, the coupling *via* $G_{q,11}$ could be enhanced by the simultaneous activation of a $G_{i,0}$ protein. This is reminiscent of the study performed by Baltensperger & Porzig (1997) in HEL cells: they found that the activation of the phospholipase C pathway by the endogenous $P2Y_2$ receptor was abolished by a $G\alpha_{16}$ antisense but was also partially inhibited by PTX. The coupling to a $G_{i,0}$ protein is consistent with the presence of a threonine residue at the end of the third intracytoplasmic loop of the human $P2Y_2$

receptor. This residue is a unique feature of $G_{i,0}$-coupled receptors (Liu *et al*., 1995). The modulation of adenylyl cyclase by the human $P2Y_2$ receptor was also studied in this paper. After 15 min stimulation, we observed a slight enhancement of the level of cyclic AMP reached under forskolin stimulation, likely to result from a crosstalk between phospholipase C and adenylyl cyclase pathways (Haslauer *et al*., 1998). The adenylyl cyclase of the $P2Y_2$ receptors expressing cells was not inhibited even at low agonist concentrations (10, 30 and 100 nM) for shorter stimulation periods (2, 5 and 10 min). This suggests that the G protein inhibited by PTX is likely a G_0 subtype.

In summary, we described here the first polymorphism affecting a P2Y family member, the human $P2Y_2$ receptor, and we analysed its distribution in a European population. Although the polymorphism generates a potential palmitoylation site, no marked pharmacological difference was observed between the two polymorphic receptors for the natural agonists we tested (UTP, ATP and AP_4A). However, there were slight differences in time-course, especially when ATPγS was used as agonist. The observation of such differences is interesting in view of the potential use of long-acting phosphorothioate nucleotides in the treatment of cystic fibrosis and chronic bronchitis (Donaldson & Boucher, 1998). Furthermore, the distribution of the two allelic variants should be investigated in clinical subsets of lung diseases, in order to evaluate if this polymorphism may act as disease modifier. Moreover, this study has revealed new pharmacological features of the human $P2Y_2$ receptor and added evidence that this receptor is coupled to at least two G proteins, probably a G_0 protein when the receptor is stimulated by low concentration of ligand and for a short period. The second G protein implicated is pertussis toxin insensitive, likely a $G_{q,11}$ protein: this coupling predominates when the $P2Y_2$ receptor is activated by high agonist concentration and/or for long duration.

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