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Slow desensitization of the human $P2Y_6$ receptor

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Abstract

The $P2Y_6$ receptor is a recently cloned P_2 receptor which displays a high sensitivity for diphosphonucleotides. In 1321N1 astrocytoma cells stably expressing this receptor, UDP induced a slow and sustained accumulation of inositol trisphosphate via a pertussis toxin-insensitive G-protein: the maximal level was only reached after 15 min and a significant response was maintained for at least 3 h. A full second response to UDP was obtained after the first 45-min stimulation, but was lost after 165 min. This slow and sustained time-course and the lack of desensitization was reproduced with ADP. UTP was unable to restimulate the $P2Y_4$ receptor, another recently cloned P_2 receptor with a preference for UTP, after the first 5-min stimulation. The $P2Y_4$ receptor is thus rapidly desensitized whereas desensitization of the $P2Y_6$ receptor is delayed. The rank order of potency of various diphosphonucleotides at the $P2Y_6$ receptor was: UDP > TDP > IDP > GDP > ADP ≫ CDP. The activity of three non-specific antagonists of P_2 receptors was characterized by the following rank order of potency: reactive blue 2 > pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) > suramin. In conclusion, the most impressive features of the human $P2Y_6$ receptor revealed by this study are the slow and sustained time-course of its activation and its high resistance to desensitization. © 1997 Elsevier Science B.V.

Keywords: Nucleotide; UDP; $P2Y_6$ receptor

1. Introduction

The P_2 receptors are subdivided into two classes: P2X receptors, which are ATP-gated cation channels, and P2Y receptors, which are G-protein-coupled heptahelical receptors (Abbracchio and Burnstock, 1994). In each of these two subfamilies, seven members have been cloned so far. The $P2Y_1$ receptor, the former P_{2Y} receptor, has been cloned in several species and is characterized by the high affinity for 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; Janssens et al., 1996). The $P2Y_2$ receptor, the former P_{2U} receptor, is characterized by the equipotency of ATP and UTP (Lustig et al., 1993; Erb et al., 1993; Parr et al., 1994; Rice et al., 1995). Both receptors are coupled to phospholipase C. The $P2Y_5$ receptor, which was previously considered an orphan receptor, has been isolated from activated chicken T lymphocytes and identified as a P2Y receptor according to binding studies (Kaplan et al., 1993; Webb et al., 1996a). Though its sequence is only distantly related to that of the other subtypes, the $P2Y_7$ receptor cloned from human erythroleukemia (HEL) cells was included in the P2Y family on the basis of a weak inositol phosphate response to ATP and because of binding data (Akbar et al., 1996). The $P2Y_3$ (Webb et al., 1996b), $P2Y_4$ (Communi et al., 1995a; Nguyen et al., 1995) and $P2Y_6$ (Chang et al., 1995; Communi et al., 1996a) receptors are also coupled to the phosphoinositide pathway, like the $P2Y_1$ and the $P2Y_2$ receptors, but have a preference for uridine over adenine nucleotides. The human $P2Y_4$ receptor is strongly activated by UTP, whereas ATP behaves as a partial agonist (Communi et al., 1996b). The chick $P2Y_3$ and the human $P2Y_6$ receptors are potently activated by UDP and to a lesser extent by ADP. These last two receptors present the same pharmacological profile of agonist activity but there is only about 60% identity between their two amino acid sequences, which is below the expected chicken-human correspondence. The cloning of these three receptors has led to the revival of the pyrimidinergic receptor concept,

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proposed by Seifert and Schultz (1989). The physiological significance of these receptors remains to be established. We have recently reported the pharmacological characterization of the human $P2Y_4$ receptor (Communi et al., 1996b). In the present study we further characterized the human $P2Y_6$ receptor, especially from the standpoint of the time-course of binding and desensitization.

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) was from Amersham (Ghent, Belgium). Dowex AG1X8 (formate form) was from Bio-Rad Laboratories (Nazareth Eke, Belgium). UDP, ADP, GDP, CDP, IDP, TDP, UTP 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 International (Natick, MA, USA).

2.2. Expression of the human $P2Y_6$ receptor in 1321N1 human astrocytoma cells

1321N1 cells were transfected with the human $P2Y_6$ coding sequence inserted into the pcDNA3 expression vector by using the calcium phosphate precipitation method as described (Communi et al., 1996a; Velu et al., 1989). The transfected cells were maintained in complete medium: DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 400 μg/ml G418. From the pool of transfected cells, 14 clones were harvested and characterized in terms of inositol trisphosphate ($insP_3$) accumulation after a 20-min stimulation with UDP (100 μM) in the presence of 10 mM LiCl (data not shown). One of these clones (named clone 1) was used for the characterization of the human $P2Y_6$ receptor. In some experiments, we also used a clone of 1321N1 cells transfected with the human $P2Y_4$ receptor (clone 11), as previously described (Communi et al., 1995a, 1996b).

2.3. Measurement of $InsP_3$ production

1321N1 cells were labeled for 24 h with 5 μCi/ml [^{3}H]inositol in inositol-free DMEM containing 5% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. 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 with the agonists in this medium for various times. The incubations were stopped by the addition of an ice-cold 3% perchloric acid solution. When tested, pertussis toxin (20 ng/ml) was added for 24 h during the labeling period time. Inositol phosphates were extracted and $InsP_3$ was isolated by chromatography on Dowex columns, as described previously (Communi et al., 1995b). The EC_{50} values were determined by curve fitting (Sigma Plot: version 2.0).

3. Results

The first functional characterization of the human $P2Y_6$ receptor has been completed recently, using a pool of 1321N1 transfected cells (Communi et al., 1996a). It appeared in this study that the human $P2Y_6$ receptor behaves as a P_2 receptor, showing a high sensitivity for UDP. The present work was performed on a cell clone (named 1) which was chosen from 14 clones harvested because it exhibited the largest $InsP_3$ response to UDP (100 μM) (data not shown). We first studied the time-course of $InsP_3$ accumulation induced by UDP or ADP in $P2Y_6$-1321N1 transfected cells (Fig. 1A). As we can see from panel A, in response to UDP (100 μM) or ADP (300 μM), the $InsP_3$ intracellular concentration increased rapidly but reached its maximal level only after 15–30 min. Thereafter, the level of intracellular $InsP_3$ decreased slowly but remained elevated for at least 1 h. An analogous time-course of $InsP_3$ accumulation was observed in response to UDP when a pool of $P2Y_6$-1321N1 cells rather than a particular clone was stimulated by UDP (data not shown). Using a similar protocol with 1321N1 cells expressing the $P2Y_4$ receptor, the time-course of the response to UTP was biphasic with an early peak at 30 s followed by a sustained stimulation of lower magnitude (Fig. 1B), as previously reported (Communi et al., 1996b). When after a first 15-min stimulation by UDP, the $P2Y_6$-expressing cells were washed twice with KRH and incubated for a further 10 min, the level of intracellular $InsP_3$ returned almost to control level,

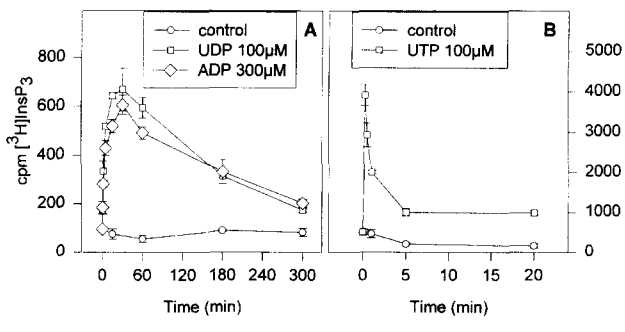

Fig. 1. Time-course of $InsP_3$ accumulation in 1321N1 cells expressing the human $P2Y_6$ receptor (A) or the human $P2Y_4$ receptor (B). [^{3}H]Inositol-labeled cells were incubated for the indicated time with 100 μM UDP or 300 μM ADP (A) or with 100 μM UTP (B). The data represent the mean ± range of duplicate points and are representative of two independent experiments.

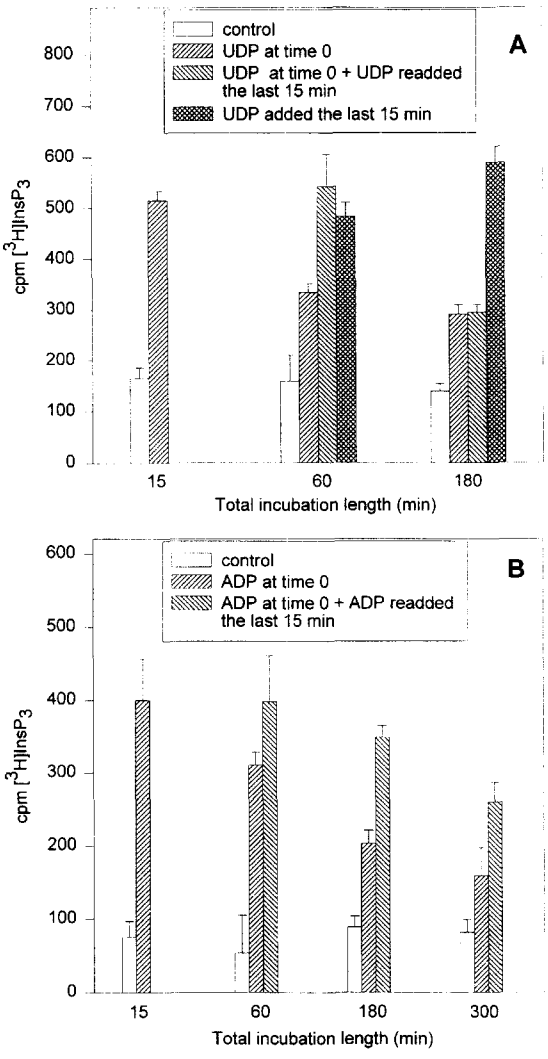

Fig. 2. Effect of successive incubations of $P2Y_6$ transfected-1321N1 cells with UDP (A) or ADP (B) on $InsP_3$ accumulation. UDP (100 μM) or ADP (100 μM) was added at time 0 and readded to some dishes 15 min before the end of the incubation. The data represent the means ± S.D. of triplicate points and are representative of two independent experiments.

indicating that the continuous presence of the agonist is needed to maintain the $InsP_3$ elevation (data not shown).

In order to investigate further the slow desensitization of the $P2Y_6$ receptor, we studied the ability of UDP or ADP to restimulate this receptor after a first stimulation (Fig. 2). When after 45 min of UDP stimulation, fresh UDP was readded to culture medium for a further 15-min period, the $InsP_3$ intracellular level returned to the maximal level observed after 15 min. When the same restimulation was done after a 165-min stimulation period, UDP was no longer able to induce a second response (Fig. 2A). Nevertheless, cells exposed to UDP for 165 min were still able to respond to carbachol through muscarinic receptors (data not shown). This high resistance to desensitization was also observed when the transfected cells were stimulated with ADP (100 μM) (Fig. 2B). Using the same protocol with 1321N1 cells expressing the $P2Y_4$ receptor,

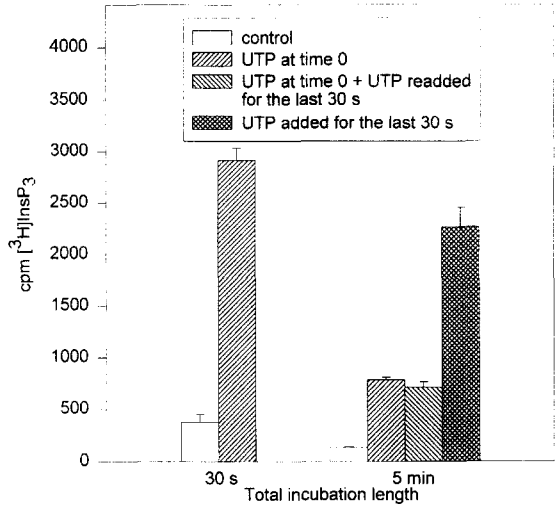

Fig. 3. Effect of successive incubations of $P2Y_4$ transfected-1321N1 cells with UTP on $InsP_3$ accumulation. UTP (100 μM) was added at time 0 and readded to some dishes 30 s before the end of the incubation. The data represent the means ± S.D. of triplicate points obtained in one representative experiment out of two.

we observed that after a first short stimulation, readdition of UTP was not able to restimulate the receptor (Fig. 3).

Full concentration-action curves were obtained at 15 min for a series of diphosphonucleotides: UDP, IDP, TDP, GDP, CDP, ADP (Fig. 4). The diphosphonucleotides tested produced the same maximal stimulation of the $P2Y_6$ receptor as UDP but with lower affinities, except CDP which behaved as a partial agonist of the $P2Y_6$ receptor. The EC_{50} values obtained for these agonists were as follows: EC_{50} UDP = 0.3 μM, EC_{50} TDP = 7.7 μM, EC_{50} IDP = 34.4 μM, EC_{50} GDP = 44.6 μM, EC_{50} ADP = 65.0 μM

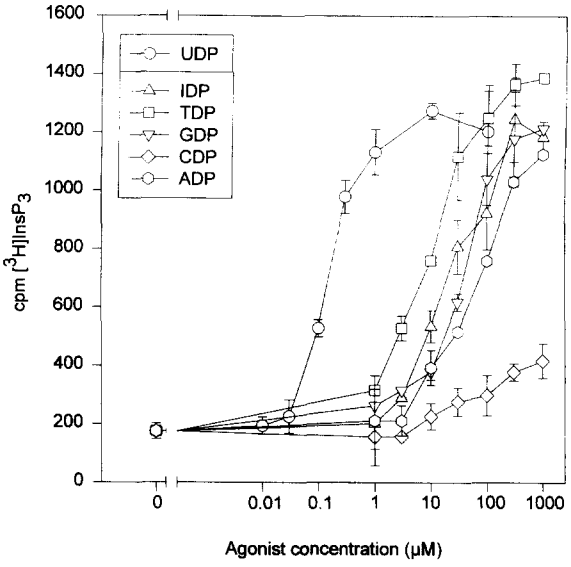

Fig. 4. Concentration-action curves of various diphosphonucleotides on $InsP_3$ accumulation in 1321N1 cells expressing the $P2Y_6$ receptor. Cells were incubated in the presence of various concentrations of nucleotides for 15 min. The data represent the means ± range of duplicate points and are representative of three independent experiments.

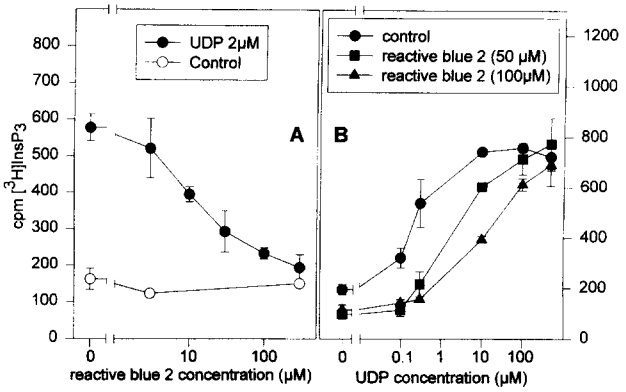

Fig. 5. Inhibitory effect of reactive blue 2 on the $InsP_3$ accumulation induced by UDP in 1321N1 cells expressing the $P2Y_6$ receptor. The cells were incubated for 15 min in the presence of various concentrations of reactive blue 2 with or without 2 μM UDP (A). The cells were incubated for 15 min with various concentrations of UDP and in the presence of 0, 50 or 100 μM of reactive blue 2 (B). The data represent the means ± S.D. of triplicate points and are representative of three independent experiments.

and EC_{50} CDP = 88.0 μM (mean values of 3 independent experiments). UMP and uridine were also tested but did not produce any effect (data not shown).

The actions of suramin, reactive blue 2 and PPADS were tested on the UDP response in our model of transfected cells. When the antagonists were tested at 100 μM on the response to 2 μM UDP, their rank order of activity was: reactive blue 2 (87% inhibition) > PPADS (69% inhibition) > suramin (27% inhibition) (mean values of 2 independent experiments) (data not shown). The IC_{50} value for reactive blue 2 was 31 μM in the presence of 2 μM UDP (mean value of three independent experiments) (Fig. 5A). Concentration-action curves of UDP in the presence or in the absence of reactive blue 2 showed that this antagonist acts in a competitive way: increasing concentrations of reactive blue 2 shifted the concentration-action curve of UDP to the right without affecting its maximal effect (Fig. 5B).

The effect of pertussis toxin (20 ng/ml; 24 h pretreatment during the labeling period) was tested with different durations of UDP (100 μM) stimulation: 1 or 15 min (Fig. 6). Pertussis toxin did not inhibit the UDP-induced $InsP_3$ accumulation at these two times.

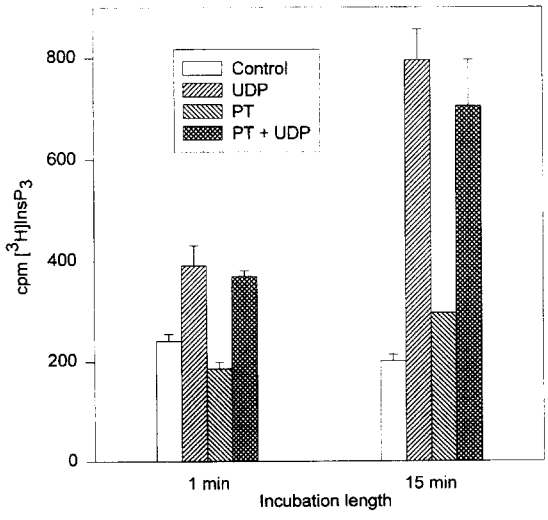

Fig. 6. Effect of pertussis toxin on the UDP response in $P2Y_6$ transfected-1321N1 cells. The cells were pre-incubated for 24 h during the labeling period in the presence or in the absence of 20 ng/ml pertussis toxin. The cells were then incubated with or without 100 μM UDP for 1 or 15 min. The data represent the means ± S.D. of triplicate points obtained in one representative experiment of two.

4. Discussion

The $P2Y_6$ receptor is a P_2 receptor which displays a high selectivity for UDP (Communi et al., 1996a; Nicholas et al., 1996) and which is natively expressed in C6-2B rat glioma cells (Lazarowski and Harden, 1994; Nicholas et al., 1996). We expressed the human $P2Y_6$ receptor in 1321N1 astrocytoma cells in order to complete the pharmacological characterization of this receptor in terms of agonist and antagonist sensitivity, desensitization and G-protein coupling. The $InsP_3$ accumulation induced by the UDP or ADP stimulation of the $P2Y_6$ receptor was characterized by a slow onset and long duration. Though stimulation was already detectable after 30 s, the maximal stimulation of $InsP_3$ was reached only after 15–30 min and a significant response was maintained for at least 3 h, in the absence of lithium (Figs. 1 and 2). This sustained level of $InsP_3$ required the continuous presence of UDP in the culture medium, since the $InsP_3$ level rapidly dropped to the control value following removal of UDP and cell washing. The slow decrease in $InsP_3$ observed after the maximum observed at 15 min seems to be due to partial degradation of the agonist, since when fresh UDP was readded during the first hour, the maximal response was restored (Fig. 2A). This time-course of $InsP_3$ stimulation is totally different from the biphasic stimulation induced by UTP in the same model of 1321N1 cells expressing the human $P2Y_4$ receptor: the maximal stimulation was reached after 30 s and was followed by a plateau of lower magnitude. These data must be compared with the time-courses of the endogeneous H_1 response to histamine and muscarinic response to carbachol (Nakahata et al., 1986; Nakahata and Harden, 1987). Indeed, incubation of non-transfected 1321N1 cells with carbachol resulted in a rapid accumulation of inositol phosphates, reaching a maximum within 30 s. This elevated level was maintained for up to 1 h. Although the initial rate of accumulation in the presence of histamine was similar to that observed with carbachol, the $InsP_3$ level returned to control within 5 min after addition of histamine. These results led the authors to conclude that the H_1 receptor is rapidly desensitized whereas the muscarinic receptor is not. Thus the $InsP_3$ responses of 1321N1 cells to histamine, carbachol, UTP

($P2Y_4$) and UDP ($P2Y_6$) have distinct and characteristic time-courses.

In view of the discrepancies observed in the $InsP_3$ time-courses, we compared the desensitization of the $P2Y_4$ and $P2Y_6$ receptors, by testing the effect of agonist readdition following a first stimulation. UTP was unable to induce a second response of the $P2Y_4$ receptor when readded after a 5-min stimulation period. In contrast, UDP and ADP could induce a full second response of the $P2Y_6$ receptor after a prior stimulation for 45 min. We can thus conclude that the $P2Y_4$ receptor is rapidly desensitized, whereas the desensitization of the $P2Y_6$ receptor is delayed. It is interesting to note here that only one potential phosphorylation site (by protein kinase C) can be found on the entire $P2Y_6$ amino acid sequence, whereas the $P2Y_4$ receptor has four different potential phosphorylation sites. The delayed desensitization of the $P2Y_6$ receptor may be the consequence of receptor internalization, whereas the rapid desensitization of the $P2Y_4$ receptor could involve the phosphorylation of the receptor by protein kinases. Of course, we cannot be sure that the $P2Y_6$ receptor exhibits the same slow rate of desensitization in the cells in which it is natively expressed.

The profile of antagonist sensitivity of the $P2Y_6$ receptor was reactive blue 2 > PPADS > suramin. Reactive blue 2 behaved as a competitive antagonist of UDP. In contrast to its intermediate effect on $P2Y_6$ receptors, PPADS is the most potent antagonist of $P2Y_1$ (Filtz et al., 1994) and $P2Y_4$ receptors (Communi et al., 1996b). PPADS is inactive on the $P2Y_2$ receptor (Brown et al., 1995).

The response to UDP was not affected by pretreatment of the 1321N1 cells expressing the $P2Y_6$ receptor by pertussis toxin, whereas we have shown earlier that pertussis toxin blocks the early response of the $P2Y_4$ receptor to UTP (Communi et al., 1996b). It seems thus that the sustained response to UDP involves a pertussis toxin-insensitive G-protein. This can be correlated with the fact that almost all the G_i-coupled receptors possess a threonine residue at the end of the third intracellular loop (Liu et al., 1995). The $P2Y_2$ and the $P2Y_4$ receptors, which are G_i-coupled receptors, share this particularity, whereas the $P2Y_1$ and $P2Y_6$ receptors, which are insensitive to pertussis toxin, do not possess this threonine residue.

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