British Journal of Pharmacology (2001) 132, 393–402 © 2001 Nature Publishing Group All rights reserved 0007–1188/01 $15.00
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Two subtypes of G protein-coupled nucleotide receptors, $P2Y_1$ and $P2Y_2$ are involved in calcium signalling in glioma C6 cells

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1 In glioma C6 cells, the stimulation of P2Y receptors by ADP, ATP and UTP initiated an increase in the intracellular Ca^{2+} concentration, in a process that involved the release of Ca^{2+} from $InsP_3$-sensitive store and the capacitative, extracellular Ca^{2+} entry. The presence of external Ca^{2+} was not necessary to elevate Ca^{2+}.

2 The rank order of potencies of nucleotide analogues in stimulating $[Ca^{2+}]_i$ was: 2MeSADP > ADP > 2MeSATP = 2ClATP > ATP > UTP. α,β-Methylene ATP, adenosine and AMP were ineffective.

3 ADP and UTP effects were additive, while actions of ATP and UTP were not additive on $[Ca^{2+}]_i$ increase. Similarly, cross-desensitization between ATP and UTP but not between ADP and UTP occurred.

4 Suramin, a non-specific nucleotide receptors inhibitor, antagonized ATP-, UTP- and ADP-evoked Ca^{2+} responses. PPADS, a selective antagonist of the $P2Y_1$ receptor-generated $InsP_3$ accumulation, decreased ADP-initiated Ca^{2+} response with no effect on ATP and UTP.

5 Pertussis toxin (PTX) reduced ADP- and ATP-induced Ca^{2+} increases. Short-term treatment with TPA, inhibited both ATP and ADP stimulatory effects on $[Ca^{2+}]_i$.

6 ADP inhibited isoproterenol-induced cyclic AMP accumulation. PTX blocked this effect, but PPADS did not.

7 RT–PCR analysis revealed the molecular identity of P2Y receptors expressed by glioma C6 cells to be both $P2Y_1$ and $P2Y_2$.

8 It is concluded that both $P2Y_1$ and $P2Y_2$ receptors co-exist in glioma C6 cells. ADP acts as agonist of the first, and ATP and UTP of the second one. Both receptors are linked to phospholipase C (PLC).

British Journal of Pharmacology (2001) **132,** 393–402

Keywords: $P2Y_1$ and $P2Y_2$ receptors; Ca^{2+} mobilization; cyclicAMP accumulation; glioma C6 cells

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; 2CIATP, 2-chloro-ATP; EGTA, [ethylene-bis(oxyethylenenitrilo]tetra-acetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; $InsP_3$, inositol 1,4,5-trisphosphate; MEM, Minimum Essential Medium; 2MeSADP, 2-methylthio-ADP; 2MeSATP, 2-methylthio-ATP; PBS, phosphate buffered saline; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid; PTX, pertussis toxin; TPA, 12-0-tetradecanoylphorbol 13-acetate

Introduction

In a variety of mammalian cells, extracellular adenine and uridine nucleotides elicit their effect either directly through interactions with cell surface P2 receptors, or through a metabolite such as adenosine, interacting with A_1-A_3 receptors (Burns, 1990; Burnstock, 1978; 1996; Ziganshin *et al*., 1994). The P2 receptors are now divided into two groups: the intrinsic ion channel P2X receptors and seven transmembrane domain, G-protein coupled, P2Y receptors (Boarder & Hourani, 1998; Burnstock 1996; Dubyak & El-Moatassim, 1993; Fredholm *et al*., 1997). Cloning and pharmacological studies revealed at least five distinct subtypes of P2Y receptors: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ (Boarder & Hourani, 1998). All of the cloned P2Y receptors activate phospholipase C (PLC) and stimulate an increase in inositol 1,4,5-trisphosphate ($InsP_3$) level. It has been found that the $P2Y_1$ receptor responds selectively to ADP compared to ATP, while ATP may act as a partial antagonist. Moreover, 2-methylthio-ATP (2MeSATP) and 2-methylthio-ADP (2MeSADP) are selective agonists at $P2Y_1$ receptor with the high potency, while UTP is not effective. On the contrary, the $P2Y_2$ receptor responds both to ATP and UTP, while 2MeSATP has no effect on its activity. These two receptors differ also in an antagonist selectivity. The $P2Y_1$ receptor-generated $InsP_3$ accumulation is competitively blocked by pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), whereas the $P2Y_2$ receptor is insensitive to this compound. $P2Y_4$ and $P2Y_6$ receptors are selective for UTP and UDP, respectively, whereas the $P2Y_{11}$

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receptor is selective for ATP but not for UTP (Boarder & Hourani, 1998; Nicholas *et al*., 1996; Webb *et al*., 1998).

While in many cell lines an activation of P2Y receptors is associated with an increase in PLC activity (Boyer *et al*., 1989), there are examples where the P2Y receptor is coupled to the inhibition of adenylyl cyclase (Boyer *at al*., 1993; 1994; 1995; 1996). The studies on glioma C6-2B and C6 cells have shown the presence of the P2Y$_1$-like receptor that is coupled to the inhibition of adenylyl cyclase but is not effective in the activation of inositol phosphate accumulation and differs in dissimilar antagonist effect from the cloned human and rat P2Y$_1$ receptors (Schachter *et al*., 1996; 1997). It has been therefore proposed that two distinct kinds of P2Y$_1$-like receptors exist: one coupled to PLC and another one, native to glioma C6 cells, negatively coupled to adenylyl cyclase. However, this interpretation has been questioned by Webb *et al*. (1996), who suggested that the same, single P2Y$_1$ receptor might be involved in different signalling pathways in different cell types. Thus, there is no conclusive evidence concerning identity of P2Y receptor subtypes in glioma C6 cells and the exact nature of the P2Y$_1$ receptor driven signalling pathways is still unclear.

It is now generally accepted that in nonexcitable cells, such as C6 glioma, biphasic capacitative Ca^{2+} signalling is mediated by the inositol system (Berridge, 1995; Putney & Bird, 1993). In this process, the initial rise in $[Ca^{2+}]_i$ results from the direct effect of $InsP_3$ on the endoplasmic reticulum store and can be observed even in the absence of extracellular Ca^{2+} (the first phase). When the extracellular Ca^{2+} is present, the depletion of this store causes opening of specific, voltage-independent Ca^{2+} channels in the plasma membrane and Ca^{2+} influx from the extracellular space (the second phase). We have shown, that in glioma C6 cells extracellular ATP increases the cytosolic Ca^{2+} concentration in a process of biphasic capacitative Ca^{2+} entry associated with $InsP_3$ accumulation and depletion of intracellular Ca^{2+} store, followed by Ca^{2+} influx from the extracellular space (Barańska *et al*., 1999; Sabała *et al*., 1997; Wójcik *et al*., 2000). However, in these cells, the effect of ADP and other nucleotide receptors agonists and antagonists on intracellular Ca^{2+} mobilization has never been examined. Therefore, the aim of this study was to characterize the functional P2Y$_1$ and P2Y$_2$ receptors in glioma C6 cells, using the measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Effects of specific pharmacological agents (agonists and antagonists of P2Y$_1$ and P2Y$_2$ receptors) on Ca^{2+} mobilization should aid in resolving the question about the nature of coupling of both receptors.

Our results indicate the presence of co-existing P2Y$_1$ and P2Y$_2$ receptors in rat glioma C6 cells with similar pharmacological properties and the same molecular identity as the cloned rat P2Y$_1$ and P2Y$_2$ receptors. We demonstrate, that both of them are linked to PLC.

Methods

Cell culture

Glioma C6 cells, obtained from the American Type Culture Collection, were cultured in Minimum Essential Medium (MEM) supplemented with 10% calf serum, penicillin (50 IU ml^{-1}), streptomycin (50 $\mu g\ ml^{-1}$) and 2 mM L-glutamine under humidified atmosphere of 5% CO_2 at 37°C. The cells were passaged when confluent by using nonenzymatic cell dissociation solution, and the medium was changed twice a week. Cells for experiments were seeded on glass coverslips and cultured for 3 days under the same conditions.

Measurement of intracellular calcium

Intracellular Ca^{2+} level was measured as described previously (Barańska *et al*., 1995) with the following modifications. Cells on coverslips were washed once with PBS and once with the solution containing (mM): NaCl 137, KCl 2.7, Na_2HPO_3 1, glucose 25, Hepes 20 (pH 7.4), $MgCl_2$ 1, 1% bovine serum albumin and 2 mM $CaCl_2$ (later referred as a standard buffer). In the experiments concerning the absence of external Ca^{2+} 500 μM EGTA was added instead of 2 mM $CaCl_2$. The cells were incubated at 37°C for 30 min in the standard buffer with 1 μM Fura-2 AM. Thereafter, the cells were washed three times with the standard buffer and coverslips were mounted in a chamber over a Nikon Diaphot inverted-stage microscope equipped with a ×40 oil-immersion fluorescence objective lens. Digital fluorescence microscopy was used to determine the changes in $[Ca^{2+}]_i$. Experiments were carried out on a video imaging system (MagiCal, Applied Imaging Ltd.). The cells were alternatively illuminated with 340 and 380 nm wavelengths of light from a xenon lamp. The emitted light was passed through a 510 nm barrier filter into an image-intensified camera (Extended ISIS, Photonic Science). The 340 and 380 nm images (256 gray levels) were software averaged and captured every 2.85 s. The 340 and 380 nm signals were examined for real changes in $[Ca^{2+}]_i$. Ratio (R) values were converted to an estimate of $[Ca^{2+}]_i$ using the following formula (Grynkiewicz *et al*., 1985):

$$[Ca^{2+}]_i = K_d \cdot \beta \cdot (R - R_{min})/(R_{max} - R)$$

R – fluorescence ratio recorded from the cell, R_{min} – fluorescence ratio in the absence of Ca^{2+}, R_{max} – fluorescence ratio in saturating concentration of Ca^{2+}, K_d – calcium dissociation constant of the fura-2, β – the ratio of the fluorescence of fura-2 Ca^{2+}-free form to the Ca^{2+}-saturated form recorded at 380 nm. Intracellular calibration was carried out by addition of 4 μM ionomycin to glioma C6 cells placed in a solution containing 2 mM Ca^{2+} ($R_{max}=2.3$) and no added calcium with 5 mM EGTA ($R_{min}=0.3$). The β value was 4.5 and K_d of 224 nM was assumed. Data processing and ratio values conversion to an $[Ca^{2+}]_i$ were carried out using Tardis V8.0 software.

All substances (agonists, antagonists, activators and inhibitors) were added as solutions in the standard buffer at final concentrations indicated in the figures.

Measurement of intracellular cyclic AMP

The adenylyl cyclase activity was estimated by measuring isoproterenol (50 μM) stimulated cyclic AMP accumulation according to Lin & Chuang (1994), assayed with the [^{3}H]-cyclic AMP assay kit. Cell pellets were assayed for protein content by a modification (Markwell *et al*., 1978) of the procedure of Lowry *et al*. (1951), and the level of cyclic AMP was expressed in picomoles per mg of protein.

Isolation of P2Y1 and P2Y2 mRNA and RT–PCR analysis

Total RNA was extracted from glioma C6 cells using TRIZOL reagent. Reverse transcription of total RNA was performed using Expand RT enzyme. Specific primes for PCR reaction were designed using 'DNA Star' software (DNA Star Inc. U.S.A.). P2Y$_1$ primers were based on unique sequences stretching bases 97–120 (AGAATGCGGCCG-GAAGAAGAGTCG, upper) and 686–666 (AGCCCAGGC-CAGCCAGGAAGG, lower) of the rat P2Y$_1$ sequence (Accession No. U22830). The estimated product length was 590 bp. P2Y$_2$ primers were based on the following unique sequences: 392–415 (CCTCCCTGCCGCTGCTGGTTTA-

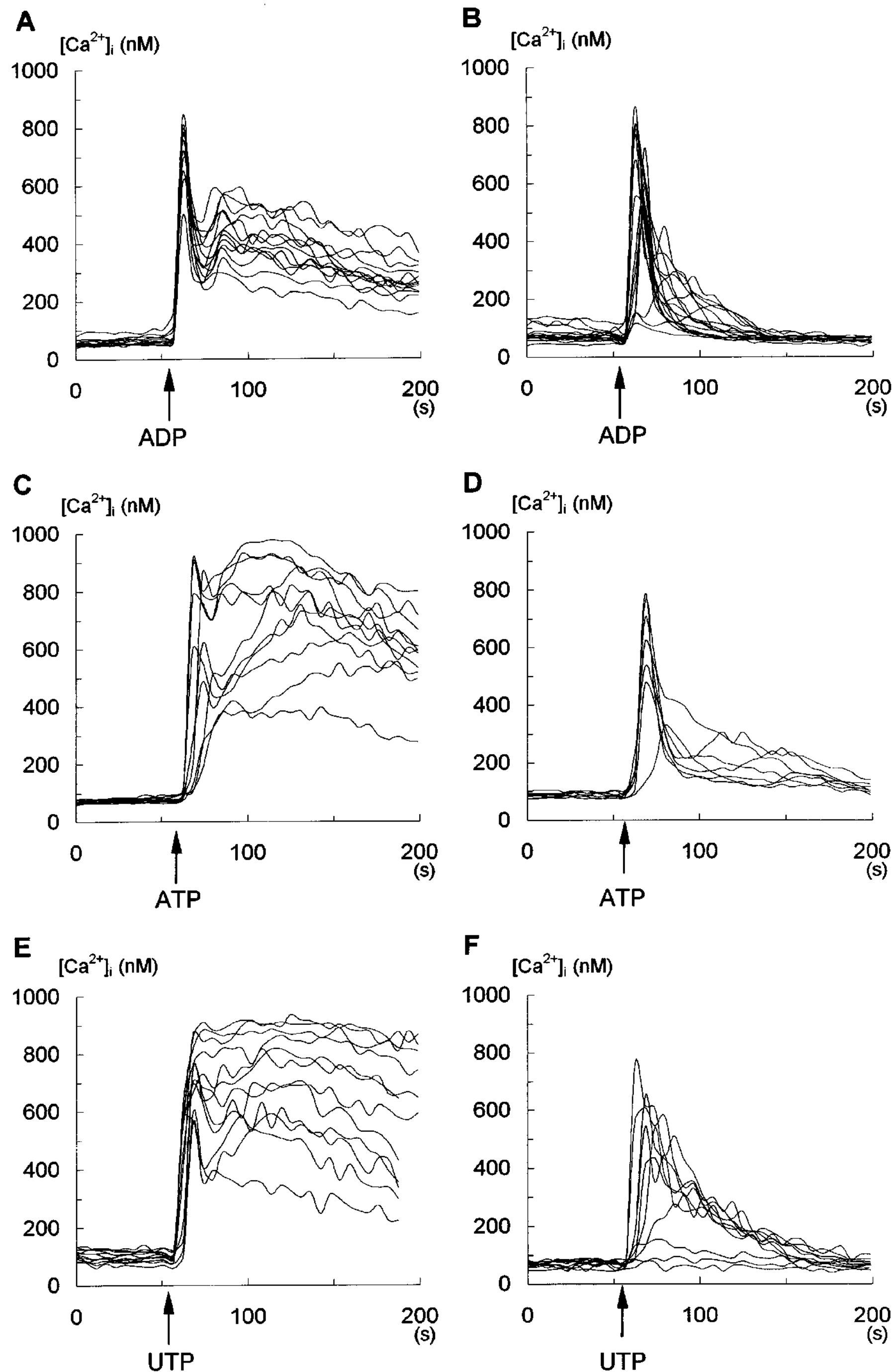

Figure 1 Effect of ADP (10 μM), ATP (100 μM) and UTP (100 μM) on changes in $[Ca^{2+}]_i$ in single glioma C6 cells. $[Ca^{2+}]_i$ was measured in Fura-2-loaded cells as described under Methods and experiments were performed either in the standard buffer containing 2 mM $CaCl_2$ (A,C,E) or without $CaCl_2$ but with 500 μM EGTA (B,D,F). Each trace in this figure represents the response of an individual cell tested during one typical experiment. Additions of ADP, ATP and UTP are indicated by arrows. Experiments in A,C,E were conducted on 10 separate occasions (number of cells tested: $n=80$). Experiments in B,D,F were conducted on five separate occasions (number of cells tested: $n=40$).

TT, upper) and 1112–1091 (TCTGTGGCGGGCTTGGC-ATCTC, lower) of the rat P2Y$_2$ sequence (Acc. No. L46865). The predicted product length was 721 bp. A hot-start PCR protocol was used involving denaturation at 95°C, 30 s, annealing at 65°C, 45 s and extension at 72°C, 2 min. Total of 35 cycles were conducted. PCR products were separated on 1% agarose gel, stained with ethidium bromide and quantified using ImageQuant and Excel programs.

Materials

Minimum Essential Medium, calf serum, antibiotics, TRIZOL reagent and phosphate buffered saline (PBS) were from Gibco BRL. ATP, ADP, AMP, UTP, BSA, EGTA, cell dissociation solution, ethidium bromide, agarose, molecular weight marker (123b), isoproterenol and phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA), were purchased from Sigma Chemical Co. Fura-2 AM was from Molecular Probes, Inc. 2MeSADP, 2MeSATP, 2-chloro-ATP (2ClATP), α,β-methylene ATP, PPADS and suramin hexasodium salt were obtained from Research Biochemicals International. PTX was purchased from Fluka. Expand RT enzyme was obtained from Boehringer Mannheim and Taq PCR Core Kit was obtained from QUIAGEN. [^{3}H]-cyclic AMP assay kit was purchased from Amersham Pharmacia Biotech.

Data analysis

Data presentation and statistical analysis were performed using ImageQuant, Tardis 8.0 and MS Excel software. Each trace shown in Figures 1 and 4 represents the response of an individual cell studied in one particular experiment, each experiment was conducted on at least five occasions and data expressed as means ± s.d. are indicated in the text. Data in Figures 2, 3 and 7 are presented as means ± s.d. from the indicated number of experiments. Traces shown in Figures 5 and 6 represent the mean value of intracellular Ca^{2+} changes

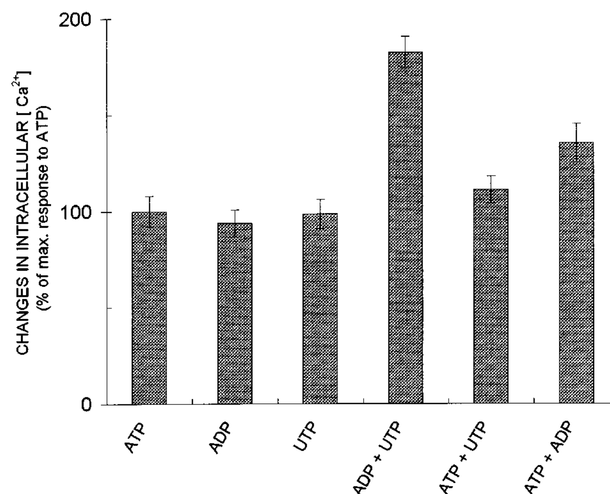

Figure 3 Additivity of ATP/ADP and UTP/ADP actions but not of ATP/UTP actions. Comparison of relative potencies of 10 μM ADP, 100 μM ATP and 100 μM UTP and their mixtures for increasing $[Ca^{2+}]_i$ are shown. Cells were treated with nucleotides in the standard buffer with 2 mM CaCl. Means ± s.d. from four experiments are shown (number of cells tested: $n=32$).

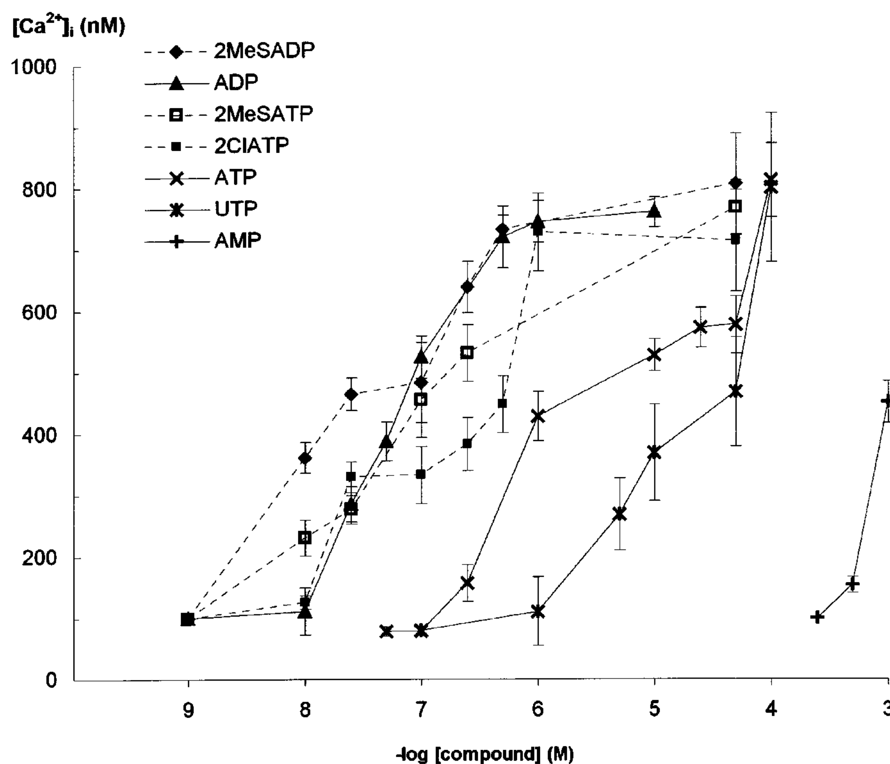

Figure 2 Concentration-dependent effect of ATP and ADP analogues on $[Ca^{2+}]_i$ in Fura-2-loaded glioma cells tested in standard buffer containing $CaCl_2$. Curves each represent the results of the five experiments (number of cells tested: $n=40$) and bars show the ± s.d.

in the indicated number of cells tested in three independent experiments.

Results

Figure 1 shows that the addition of ADP, ATP and UTP induced rapid changes in $[Ca^{2+}]_i$ in single, intact glioma C6 cells, in both the presence (Figure 1A,C,E) and absence (Figure 1B,D,F) of extracellular Ca^{2+}. When extracellular medium contained 2 mM $CaCl_2$, the addition of ADP, ATP and UTP caused a rapid rise in $[Ca^{2+}]_i$, reaching a maximum at few seconds. This increase was associated with the depletion of intracellular Ca^{2+} stores (the first phase) and was followed by the sustained elevation in $[Ca^{2+}]_i$ (the second phase), which was a result of a subsequent influx of extracellular Ca^{2+} to the cells. Traces shown in Figure 1 represent the responses of individual cells studied in one particular experiment. The statistical analysis (mean value $\pm$s.d. data from 10 separate experiments and for the indicated number of cells *n*) revealed that the initial peak of Ca^{2+} elevation following exposure to 100 μM ATP was 811 ± 60 nmol ($n=80$) and to 100 μM UTP was 799 ± 121 nmol ($n=80$). ADP was more effective and in concentration 10 μM induced a similar huge rise in $[Ca^{2+}]_i$ with the initial peak of Ca^{2+} amounted to 760 ± 75 nmol ($n=80$). However, the kinetics of this ADP-evoked Ca^{2+} changes differed from those evoked by ATP and UTP. The ATP-and UTP-induced Ca^{2+} elevation, started with an initial peak response was followed by a long sustained plateau phase (Figure 1C,D) which, in most cases, was still above basal level even after 15 min exposure to both nucleotides (not shown). In contrast, the response to ADP was more transient. The initial Ca^{2+} peak declined distinctly towards basal line; there was a significant decrease in the level measured at 16 s after the ADP addition (Figure 1A). This peak was followed by the second, sustained phase that declined to the basal level at 300 s in almost all cells examined (84%, $n=80$) (see Figure 6A). In the absence of extracellular Ca^{2+} (500 μM EGTA), 10 μM ADP, 100 μM ATP and 100 μM UTP resulted only in the initial transient rise in $[Ca^{2+}]_i$ which declined to the basal level (the first phase), indicating that this phase of the cytosolic Ca^{2+} increase was indeed caused by mobilization of intracellular Ca^{2+} stores (Figure 1B,D,F). The maximal values of Ca^{2+} elevation after exposure of cells to ADP amounted to 711 ± 120 nmol ($n=40$) (Figure 1B), ATP to 676 ± 103 nmol ($n=40$) (Figure 1D), and UTP to 597 ± 59 nmol ($n=40$) (Figure 1F). Essentially, all cells (95%, 152 out of 160) responded to 10 μM ADP, while most responded to 100 μM ATP (85%, 204 out of 240) and 100 μM UTP (71%, 113 out of 160).

In order to characterize pharmacological properties of nucleotide receptors generating Ca^{2+} mobilization in glioma C6 cells, the increase in $[Ca^{2+}]_i$ in response to various nucleotide analogues was measured. Figure 2 shows concentration-effect curves established for ATP and ADP analogues. The calculated EC_{50} values for Ca^{2+} mobilization induced by these agonists were as follows (in nM) 2MeSADP 20, ADP 84, 2MeSATP 148, 2ClATP 183; (in μM): ATP 4.5, UTP 15.8 and AMP 664. This relative potency: 2MeSADP > ADP > 2MeSATP = 2ClATP > ATP > UTP, was consistent with the order of potency expected for P2Y receptors. AMP was practically ineffective (Figure 2). P2X nucleotide receptors selective agonist, α,β-methylene ATP, and adenosine, interacting with A_1-A_3 receptors, had no effect on the increase in $[Ca^{2+}]_i$ in glioma C6 cells when assayed at concentrations up to 1 mM (not shown). It is worth noting that 2MeSATP, 2MeSADP and 2ClATP increased $[Ca^{2+}]_i$ in a manner typical for ADP (Figure 1), i.e. with the initial Ca^{2+} peak (the first phase) distinctly separated from the second phase that declined very quickly towards basal level (not shown).

Figure 3 shows that a mixture of 10 μM ADP and 100 μM UTP raised $[Ca^{2+}]_i$ level in an additive manner. On the contrary, 100 μM ATP and 100 μM UTP did not increase $[Ca^{2+}]_i$ to a level higher than those observed for ATP and UTP alone, indicating that their action was nonadditive. On

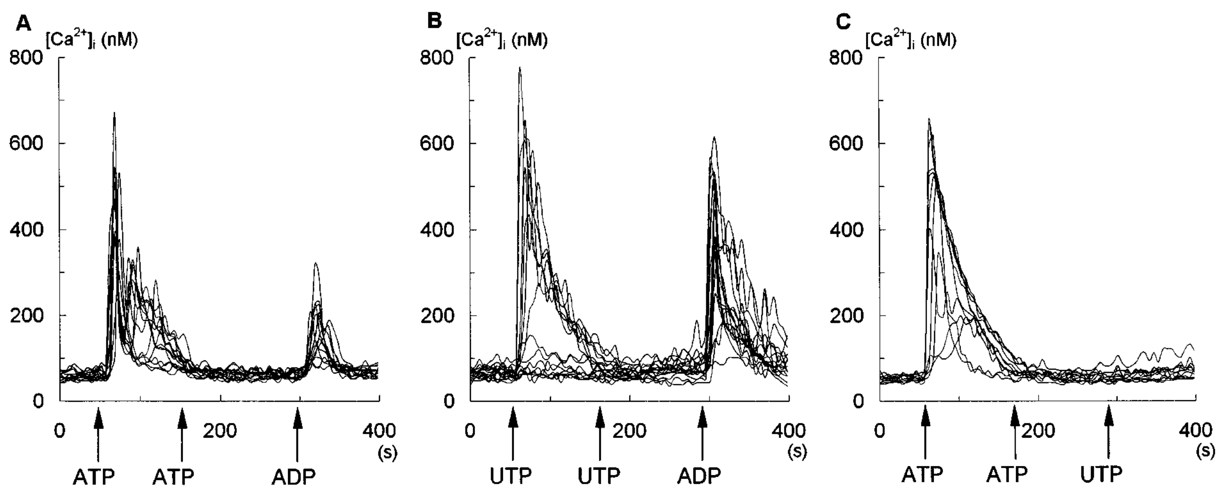

Figure 4 Effect of the sequential addition of ATP (100 μM), UTP (100 μM) and ADP (10 μM) on glioma C6 cells measured in the standard buffer without $CaCl_2$ and with 500 μM EGTA. Each trace in this figure represents the response of an individual cell tested during one typical experiment. Additions of ADP, ATP and UTP are indicated by arrows. Experiments in A–C were conducted on three separate occasions (number of cells tested: $n=24$).

the other hand, the mixture of 10 μM ADP and 100 μM ATP produced only partial additive effect on the increase in $[Ca^{2+}]_i$. The similar results were obtained in experiments presented in Figure 4, where the cells were treated with sequential additions of nucleotides. As is shown, 100 μM ATP and 100 μM UTP added to the cells after the previous addition of the same concentration of these nucleotides had no further effect on the cytosolic Ca^{2+} level. Such ATP and UTP treated cells were capable to respond to ADP, although the ADP-induced rise in $[Ca^{2+}]_i$ was larger in the cells first treated with UTP (Figure 4B), than in those treated with ATP (Figure 4A). In contrast, the cells treated with ATP failed to respond to UTP (Figure 4C), indicating that cross-desensitization between ATP and UTP occurred. These data suggested that ATP and UTP exerted their effects on a common subtype of the P2Y receptor, while ADP is recognized by another one.

The effect of P2Y nucleotide receptor antagonists on ADP-, ATP- and UTP-induced $[Ca^{2+}]_i$ increase is presented in Figure 5. Suramin (1 mM), a broad spectrum P2 receptors inhibitor known to antagonize both P2Y$_1$ and P2Y$_2$ receptors, distinctly blocked the effect of ADP, ATP and UTP on $[Ca^{2+}]_i$ by 90, 82 and 75%, respectively, compared to the control glioma C6 cells (Figure 5A,D,G versus B,E,H). On the other hand, PPADS (100 μM), a selective P2Y$_1$ but not P2Y$_2$ antagonist, strongly decreased the Ca^{2+} response to ADP (by 94%) (Figure 5C) but was without an influence on changes produced by ATP (Figure 5F) and UTP (Figure 5I). These results suggested the presence of both, P2Y$_1$ and P2Y$_2$ receptors, with ADP as agonist of the first, and ATP and UTP of the second one.

To examine the nature of coupling between P2Y receptors and intracellular Ca^{2+} elevation, Ca^{2+} responses to nucleotides in the control cells (Figure 6A,B) were compared to

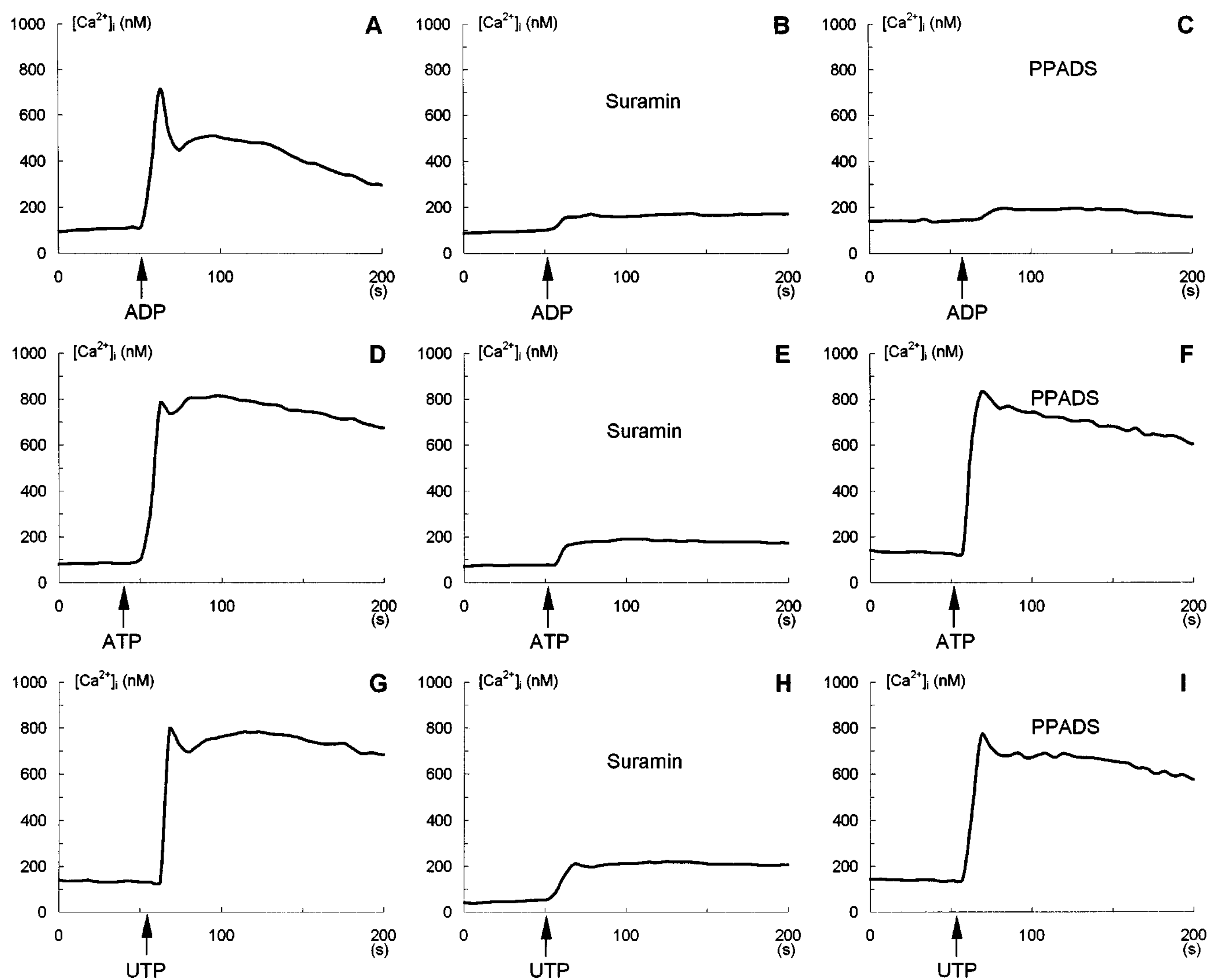

Figure 5 Effect of P2Y nucleotide receptor antagonists, suramin and PPADS, on ADP-, ATP- and UTP-induced changes in $[Ca^{2+}]_i$ in glioma C6 cells. $[Ca^{2+}]_i$ was measured as described under Methods and experiments were performed in the standard buffer containing 2 mM $CaCl_2$. A,D,G: Control cells, (A) 10 μM ADP added, (D) 100 μM ATP added, (G) 100 μM UTP added. B,E,H: Cells were treated with 1 mM suramin for 10 min prior to addition of 10 μM ADP (B), 100 μM ATP (E) and 100 μM UTP (H). G,F,I: Cells were treated with 100 μM PPADS for 2 min prior to addition of 10 μM ADP (C), 100 μM (F) and 100 μM UTP (I). Additions of ADP, ATP and UTP are indicated by arrows. Each trace represents a mean value for 24 cells. Each experiment was conducted on three separate occasions.

those in the cells pretreated with PTX (500 ng ml^{-1}), or 100 nM TPA (Figure 6C,D). In experiments with PTX, the toxin was added to the culture medium 4 h before the experiments (Figure 6), but the similar results were obtained when the cells were treated 24 h with 100 ng ml^{-1} (not shown). As is shown, PTX reduced the 10 μM ADP- and 100 μM ATP-evoked $[Ca^{2+}]_i$ increase by 88 and 59%, respectively (Figure 6C,D). Figure 6 also shows that short-term treatment (5 min) with 100 nM TPA strongly diminished the stimulatory effect of 10 μM ADP and 100 μM ATP on $[Ca^{2+}]_i$ increase by 70 and 89%, respectively, suggesting protein kinase C (PKC) -inhibitory feedback (Figure 6C,D; dashed lines).

To check whether in the cell line studied herein ADP could inhibit adenylyl cyclase, experiments with ADP effect on cyclic AMP accumulation were performed. The level of cyclic AMP increased about 140 times (from 8 ± 3 to 1115 ± 195 pmol mg^{-1} protein) within 5 min in response to 50 μM isoproterenol. ADP, used at the same concentration (10 μM) as that inducing a large increase in $[Ca^{2+}]_i$, inhibited the isoproterenol-stimulated cyclic AMP synthesis by about 80%. This effect was significantly reversed by 24 h pretreatment of the cells with 10 ng ml^{-1} or 100 ng ml^{-1} PTX. In contrast, 100 μM PPADS did not abolish the ADP-induced cyclic AMP inhibition (Figure 7).

To confirm the identity of P2Y nucleotide receptors in glioma C6 cells, polymerase chain reaction on reverse transcribed total mRNA was employed. Primers were selected to cover unique sequences of rat $P2Y_1$ and $P2Y_2$ receptors. The estimated product size was 590 and 721 bp, respectively. Figure 8 shows that mRNAs of both receptors are expressed in glioma C6 cells. The experiments on five independent cell colonies demonstrated that $P2Y_1$ and $P2Y_2$ mRNA expression levels were not very much different, with $P2Y_1$ mRNA expression slightly, about 1.5 fold, higher than that of $P2Y_2$.

Discussion

In this study we show that ADP, ATP and UTP initiate a large Ca^{2+} response consistent with the typical, biphasic capacitative model of Ca^{2+} entry associated with the stimulation of PLC and depletion of intracellular Ca^{2+} stores. The similar response was observed with several other

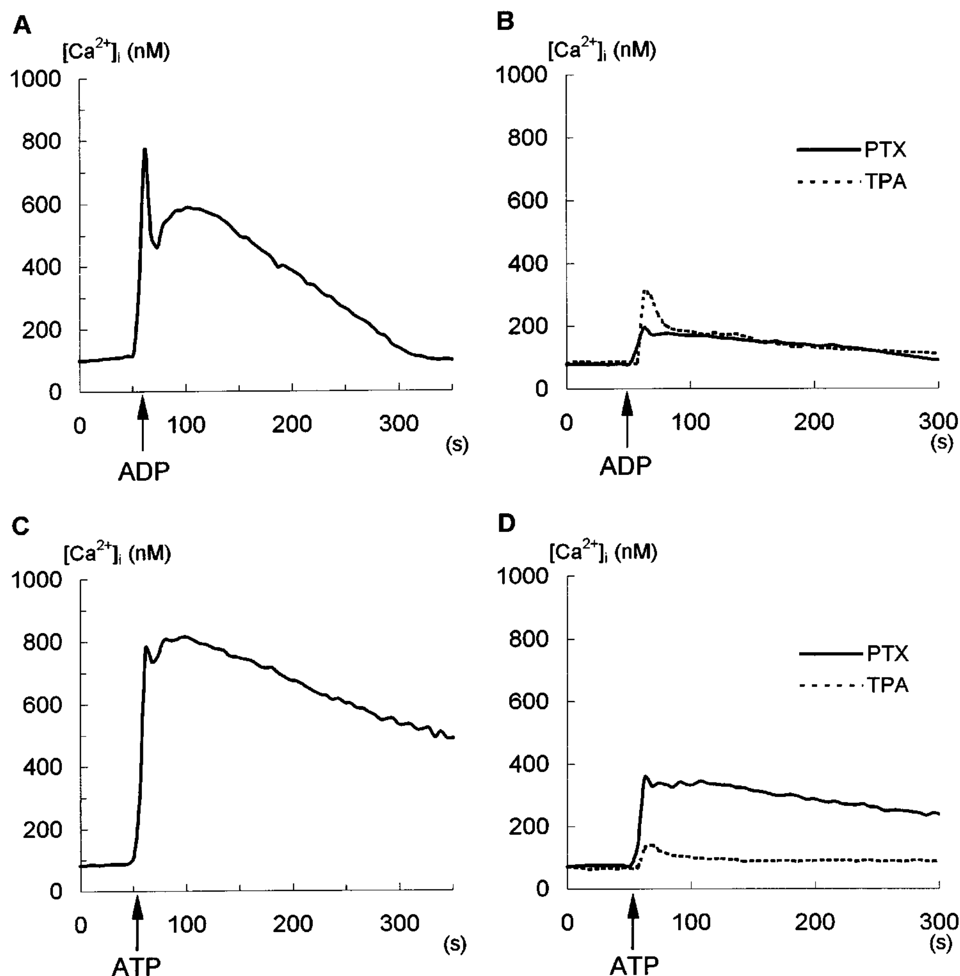

Figure 6 Modulation of ADP- and ATP- evoked Ca^{2+} signals in glioma C6 cells by PTX and phorbol ester, TPA, measured in the standard buffer containing 2 mM $CaCl_2$. A,C: Control cells, (A) 10 μM ADP added, (C) 100 μM ATP added. B,D: Cells were treated 4 h with 500 ng ml^{-1} PTX (solid lines), or 5 min with 100 nM TPA (dashed lines) prior to addition of 10 μM ADP (B) or 100 μM ATP (D). Additions of ADP and ATP are indicated by arrows. Each trace represents a mean value for 24 cells. Each experiment was conducted on three separate occasions.

metabotropic P2Y nucleotide receptor agonists such as 2MeSATP, 2MeSADP and 2ClATP, while no increase in $[Ca^{2+}]_i$ was observed with α,β-methylene ATP, a selective agonist of the inotropic P2X receptors. Adenosine and AMP were ineffective in increasing $[Ca^{2+}]_i$. These and other results of this study, concerning: (i) the apparent rank order of potency for increasing $[Ca^{2+}]_i$, (ii) the effect of antagonists, (iii) the additive actions of ADP and UTP with the lack of additivity of ATP and UTP, (iv) the response of ATP-desensitized cells to ADP but not to UTP, and (v) the RT–PCR reaction, provide a strong support for the presence of two subtypes of the P2Y receptor in glioma C6 cells: the P2Y$_1$ for ADP and the P2Y$_2$ for ATP and UTP.

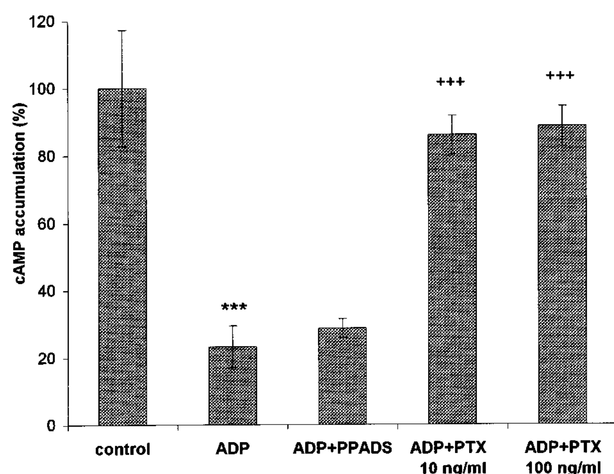

Figure 7 Effect of PTX and PPADS treatment on the inhibition induced by ADP of the isoproterenol-elicited cAMP response. The cells were treated with 50 μM isoproterenol in the absence or presence of ADP (10 μM) for 5 min at 37°C in the standard buffer. PTX (10 or 100 ng ml^{-1}) was added to the culture medium 24 h and PPADS (100 μM) 2 min before the experiment. Cyclic AMP levels were assayed with the [^{3}H]-cyclic AMP kit. The response to isoproterenol in the absence of added ADP was taken as 100%. The data are means$\pm$s.d. from four independent experiments done in duplicate. Statistical significance of differences, when comparing isoproterenol versus isoproterenol and ADP treated cells is $***P<0.001$, and isoproterenol and ADP versus isoproterenol and ADP and PTX (10 and 100 ng ml^{-1}) treated cells is $+++P<0.001$ (Student's *t*-test).

The presence of the P2Y$_2$ receptor stimulated by both ATP and UTP has been documented in a variety of cells. Among others, it has been found in brain capillary endothelial cells (Albert *et al.*, 1997; Feolde *et al.*, 1995), aortic endothelial cells and in platelets (for review, see Boarder & Hourani, 1998; Boeynaems *et al.*, 2000; Kunapuli, 1998). In glioma C6 cells, ATP has been reported to mediate phosphoinositide turnover and stimulate phosphoinositide hydrolysis (Kitanaka *et al.*, 1992; Lin & Chuang, 1994). We have also demonstrated that in these cells ATP treatment generates InsP$_3$ and simultaneously results in increases $[Ca^{2+}]_i$ due to the PLC- mediated release from intracellular stores (Sabała *et al.*, 1997). Thus, our present results confirm previous data indicating that ATP and UTP act on the P2Y$_2$ receptor coupled to PLC and elevated $[Ca^{2+}]_i$.

In contrast to the PLC activation stimulated by ATP and UTP, the receptor that responds to ADP in rat glioma C6 cells had been to date believed not to be coupled to PLC. Such a notion had been supported by the observation that ADP did not cause the detectable increase in InsP$_3$. On the other hand, the cloned rat P2Y$_1$ receptor produced such effect in response to ADP (Boarder & Hourani, 1998; Boyer *et al.*, 1994; 1995; 1996; Pianet *et al.*, 1989; Schachter *et al.*, 1996; 1997).

The present study provide several lines of evidence that the PLC-coupled P2Y$_1$ receptor may exist in glioma C6 cells. First, RT–PCR analysis revealed that the P2Y$_1$ receptor mRNA is localized to these cells. Second, 2MeSATP, 2MeSADP, ADP and 2ClATP, the specific agonists of the P2Y$_1$ receptor were most potent and, PPADS, the selective antagonist of this PLC-coupled-generated InsP$_3$ accumulation receptor completely blocked ADP-evoked $[Ca^{2+}]_i$ increases. Third, Ca^{2+} rises induced by ADP were distinctly diminished by a phorbol ester, TPA. It has been documented that TPA activates PKC that exerts negative feedback regulation on receptor-coupled PLC and, due to that, inhibits phosphatidylinositol 4,5-bisphosphate (PIP$_2$) hydrolysis, InsP$_3$ production and Ca^{2+} mobilization (Ryu *et al.*, 1990). It has been found that TPA *via* PKC also inhibits ATP-evoked $[Ca^{2+}]_i$ raises (see this study and Chen & Lin, 1999; Czajkowski & Barańska, 1999). Fourth, ADP and other nucleotide analogues initiated biphasic capacitative Ca^{2+} responses and the presence of external Ca^{2+} was not necessary to elevate

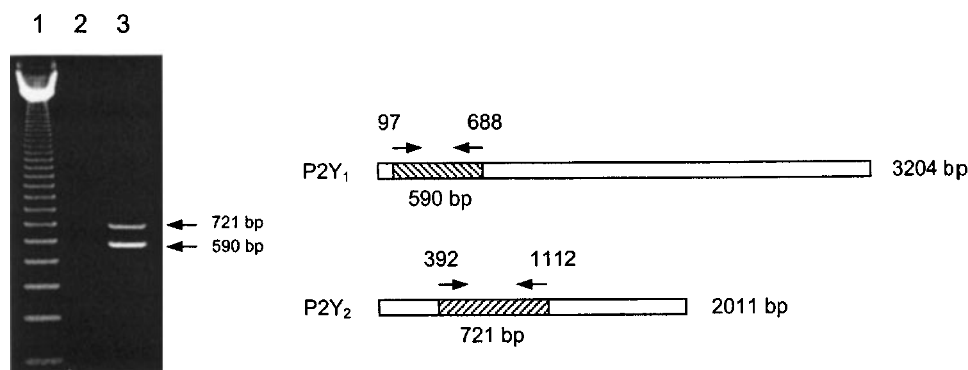

Figure 8 Detection of P2Y$_1$ and P2Y$_2$ mRNA with RT–PCR reaction. A 590 and 721 bp amplification products (corresponding to P2Y$_1$ and P2Y$_2$ mRNAs, respectively) were analysed on 1% agarose gel and visualized by ethidium bromide staining. Lane 1, molecular weight marker 123 bp; Lane 2, control reaction in the absence of cDNA template; Lane 3, PCR products obtained in separate reactions but run together for better visualization.

$[Ca^{2+}]_i$. However, in contrast to ATP and UTP, the second phase of this response was transient, quickly reduced to the basal level and 4–5 min after addition of agonists no more visible. Thus, one can expect that ADP-evoked $InsP_3$ accumulation is also transient and too short to be estimated. Brain endothelial cells have P2Y$_1$-like receptors which similarly stimulate an increase in $[Ca^{2+}]_i$ in the absence of detectable increase in $InsP_3$ levels (Frelin *et al*., 1993; Albert *et al*., 1997).

In addition to its effect on the Ca^{2+} increase, ADP is known to inhibit cyclic AMP accumulation in rat C6 glioma cells and its inhibitory effect is reversed by PTX but insensitive to PPADS (see this study and Boyer *et al*., 1994; 1995; 1996; Pianet *et al*., 1989; Schachter *et al*., 1996; 1997). Schachter *et al*., (1996; 1997) suggested that the P2Y$_1$ receptor coupled to PLC is distinct from an endogenous P2Y$_1$-like receptor in glioma C6 cells. This interpretation has been questioned by Webb *et al*. (1996), who purified P2Y receptor cDNA from C6-2B cells, known to possess a P2Y receptor negatively coupled to adenylyl cyclase, and found that sequence analysis of the entire coding region revealed 100% identity to the cloned rat P2Y$_1$ receptor. The authors proposed that the same, single receptor might be coupled to the inhibition of adenylyl cyclase in some cells and to the activation of PLC in others.

Thus, two possible interpretations of the obtained results could be advanced. First that the single P2Y$_1$ receptor is involved in both activities: an inhibition of adenylyl cyclase (PPADS-insensitive) and an activation of PLC (PPADS-sensitive). According to this suggestion, this receptor might be dually coupled to both G_q (PLC) and G_i (adenylyl cyclase) as it has been recently shown for other P2Y receptors (Boeynaems *et al*., 2000). On the other hand, there is growing evidence that many receptors coupled to G_i (e.g., adenosine A_1) can simultaneously modulate adenylyl cyclase and activate PLC *via* $G_i\alpha$ and $\beta\gamma$ subunits, respectively (Bugrim, 1999; Clapham & Neer, 1997; Selbie & Hill, 1998). However, although the present study shows that ADP-evoked Ca^{2+} elevation can be reduced by PTX, this blockade does not prove a direct G_i involvement in this signalling pathway since G_i and G_o are usually present at much higher amounts than other G protein and might affect G_q-related signalling. The second possibility is the presence of two ADP receptors in glioma C6 cells: the cloned P2Y$_1$ receptor coupled to PLC and yet-to-be cloned P2Y$_1$-like receptor negatively coupled to adenylyl cyclase. The presence of two ADP receptors, one coupled to PLC *via* Gq and other, yet uncharacterized, coupled to G_i and associated with adenylyl cyclase, has been postulated in platelets (Boeynaems *et al*., 2000; Gachet *et al*., 1997). Further studies should elucidate this problem and answer these still open questions.

In conclusion, our results indicate that both P2Y$_1$ and P2Y$_2$ receptors are expressed and linked to PLC and Ca^{2+} release in glioma C6 cells. We show that in these cells ADP has two activities: it stimulates PLC and inhibits adenylyl cyclase. The cross-talk between these pathways most probably leads to a multiple network of simultaneous regulation of $InsP_3$ and cyclic AMP mediated signalling.

This study was supported by a grant No 4PO5A04408 from the State Committee for Scientific Research (KBN, Poland).

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(Received August 18, 2000
Revised November 6, 2000
Accepted November 14, 2000)