ADP-evoked phospholipase C stimulation and adenylyl cyclase inhibition in glioma C6 cells occur through two distinct nucleotide receptors, $P2Y_1$ and $P2Y_{12}$

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Abstract In this study we characterized the subtypes of nucleotide P2Y receptors that respond to ADP in glioma C6 cells. Direct visualization of phosphatidylinositol 4,5-bisphosphate at the cell surface revealed that extracellular ADP activates phospholipase C (PLC). Knock-down of $P2Y_1$ receptor with antisense oligonucleotide, as well as treatment with MRS2179 and pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid ($P2Y_1$ antagonists), attenuates receptor-mediated PLC activity. Adenylyl cyclase inhibition by ADP remains unchanged under these conditions. Reverse transcription-PCR analysis showed that $P2Y_{12}$ receptor is expressed in C6 cells. We therefore conclude that, in glioma C6 cells, two P2Y receptor subtypes are present: $P2Y_1$, coupled to PLC, and $P2Y_{12}$, negatively coupled to adenylyl cyclase.

Key words: Nucleotide receptor; $P2Y_1$; $P2Y_{12}$; Phospholipase C; Adenylyl cyclase; Glioma C6 cell

1. Introduction

Extracellular adenine and uridine nucleotides act through two families of membrane bound P2 receptors: P2Y subtypes, all of which are G protein-coupled receptors, generally coupled to phospholipase C (PLC), and P2X subtypes which are ligand-gated channels [1]. Within the family of P2Y receptors, $P2Y_1$ responds selectively to ADP and is partially antagonized by ATP, while the $P2Y_2$ receptor responds both to ATP and UTP [2]. $P2Y_1$ receptor was first cloned from chick [3], and then successively from turkey [4], rat and mouse [5], bovine [6] and human tissues [7]. All of them activate PLC.

While extracellular ADP has been reported to stimulate PLC activity in various cells, with the resulting production of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol [8], there are examples where ADP activates P2Y receptor coupled to the inhibition of adenylyl cyclase [9–11]. Studies on glioma C6-2B and C6 cells have shown the presence of a $P2Y_1$-like receptor. This receptor inhibits adenylate cyclase with similar agonists but dissimilar antagonists as $P2Y_1$ receptor. Also, it is believed not to be coupled to PLC [9,11–14].

We have recently found that glioma C6 cells express two types of nucleotide receptors, of molecular and pharmacological identity consistent with $P2Y_1$ and $P2Y_2$, which both lead to PLC activation [15]. We have provided several lines of evidence that ADP acts on the $P2Y_1$ receptor coupled to PLC and elevated cytosolic Ca^{2+}. It has been demonstrated that, in glioma C6 cells, ADP has two activities: it stimulates PLC and mobilizes $[Ca^{2+}]$, and inhibits adenylyl cyclase. This effect was pertussis toxin (PTX)-sensitive, suggesting G_i protein involvement. In conclusion, we supposed that either the single $P2Y_1$ receptor was involved in both activities, or that two receptors were present in these cells: the cloned $P2Y_1$ receptor and yet-to-be cloned $P2Y_1$-like receptor coupled to adenylyl cyclase [15]. Most recently, a P2Y receptor subtype that couples to the inhibition of adenylyl cyclase in rat blood platelets, previously termed $P2T_{AC}$, has been cloned and designated $P2Y_{12}$ [16]. The presence of this receptor has also been reported in C6-2B glioma cells [17]. Therefore, the aim of this study was to differentiate whether ADP acts on one (coupled to different G proteins) or two distinct P2Y receptors in glioma C6 cells and to characterize the functional $P2Y_1$-like receptor associated with adenylyl cyclase.

In the present study we first confirm our previous results showing that ADP stimulates PLC by using the direct visualization of phosphatidylinositol 4,5-bisphosphate (PIP_2) at the cell surface. Second, using an antisense oligonucleotide technique, we demonstrate that knock-down of the $P2Y_1$ receptor causes a decrease in the $P2Y_1$ mRNA level and loss of the receptor-evoked PLC activity, whereas the ADP-generated inhibition of adenylyl cyclase remains unchanged. Third, we reveal, using reverse transcription-PCR (RT-PCR) analysis, that the $P2Y_{12}$ receptor is expressed in glioma C6 cells. Therefore, we conclude that, in these cells, two ADP-responding receptors coexist: $P2Y_1$ linked to PLC, and $P2Y_{12}$ negatively coupled to adenylyl cyclase through G_i.

2. Materials and methods

2.1. Materials

Minimum essential medium (MEM), calf serum, antibiotics, Lipofectamine 2000, Trizol reagent and phosphate-buffered saline (PBS) were from Gibco BRL. TRI reagent, ADP, bovine serum albumin

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Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; GFP, green fluorescent protein; $InsP_3$, inositol 1,4,5-trisphosphate; MEM, minimum essential medium; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid; PTX, pertussis toxin

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(BSA), EGTA, ethidium bromide, agarose, molecular weight marker (123 bp) were purchased from Sigma Chemical Co. Fura-2/AM was from Molecular Probes, Inc. Pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) was obtained from Research Biochemicals International. Expand RT enzyme was purchased from Roche. Taq PCR core kit, Qiaex and EndoFree kits were obtained from Qiagen. TransFast reagent was obtained from Promega. MRS2179 tetraammonium salt was from Tocris. Cyclic AMP (cAMP) assay kit was from Amersham Pharmacia Biotech.

2.2. Cell culture

Glioma C6 cells (passages 40–60) were cultured in MEM supplemented with 10% (v/v) calf serum, penicillin (50 IU/ml), streptomycin (50 μg/ml) and 2 mM L-glutamine under a humidified atmosphere of 5% CO_2 at 37°C.

2.3. Measurement of intracellular calcium

Intracellular Ca^{2+} levels were measured as described previously [18] with the following modifications. Cells on coverslips were washed once with PBS and once with the solution containing: 137 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO_3, 25 mM glucose, 20 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 1% (v/v) BSA and 2 mM $CaCl_2$ (standard buffer). 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 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 nm and 380 nm images (256 gray levels) were software averaged and captured every 2.85 s. The 340 nm and 380 nm signals were examined for real changes in $[Ca^{2+}]_i$. Ratio (R) values were converted to an estimate of $[Ca^{2+}]_i$ according to Grynkiewicz et al. [19]. Data processing and ratio value conversion to $[Ca^{2+}]_i$ were carried out using Tardis V8.0 software.

2.4. Measurement of intracellular cAMP

The adenylyl cyclase activity was estimated by measuring isoproterenol (50 μM)-stimulated cAMP accumulation according to Lin and Chuang [20], assayed with the [^{3}H]cAMP assay kit. Cell pellets were assayed for protein content by a modification [21] of the procedure of Lowry et al. [22], and the level of cAMP was expressed in pmol per mg of protein.

2.5. Isolation of $P2Y_1$ and $P2Y_{12}$ mRNA and RT-PCR analysis

Total RNA was extracted from glioma C6 cells using TRI reagent. Reverse transcription of total RNA (1 μg) was performed using Expand RT enzyme. Specific primers for the PCR reaction were designed using 'DNA Star' software (DNA Star Inc. USA). $P2Y_1$ primers were based on unique sequences stretching bases 97–120 (5′-AGA ATG CGG CCG GAA GAA GAG TCG-3′, upper) and 686–666 (5′-AGC CCA GGC CAG CCA GGA AGG-3′, lower) of the rat $P2Y_1$ sequence (accession no. U22830). The estimated product length was 590 bp. $P2Y_{12}$ primers were based on the following unique sequences: 533–554 (5′-AAA CTT CCA GCC CCA GCA ATC T-3′, upper) and 1021–1002 (5′-CAA GGC AGG CGT TCA AGG AC-3′, lower) of the rat $P2Y_{12}$ sequence (accession no. L46865). The predicted product length was 489 bp. A hot-start PCR protocol was used involving denaturation at 95°C, annealing at 65°C ($P2Y_1$) or 56°C ($P2Y_{12}$) and extension at 72°C. A total of 30 cycles were conducted. PCR products were separated on 1% agarose gels, stained with ethidium bromide and quantified using ImageQuant and Excel programs.

2.6. Construction of fusion protein

Total RNA was prepared from mouse brain using Trizol reagent. Reverse transcription of total RNA was performed using Expand RT enzyme. The pleckstrin homology (PH) domain of $PLC\delta_1$ (1–170) was amplified with Taq PCR core kit (Qiagen) and the following primer pair:

Upper: 5′-ATATAGATCTAGGCCGCTTGGTGATTTT-3′

Lower: 5′-ATATGTCGACCCTCCCGCTGCTGATGC-3′

A hot-start PCR protocol was used involving denaturation at 95°C, annealing at 60°C and extension at 68°C. A total of 35 cycles were conducted. PCR products were separated on 1% agarose gels, and isolated using Qiaex gel extraction kit. The PCR product was subcloned in-frame into the *Bgl*II site of green fluorescent protein (GFP) mammalian expression vector pS65T-C1 (Clontech). The final product encoding the PH domain of mouse $PLC\delta_1$ fused to the C-terminus of GFP was used for transfection.

2.7. Glioma C6 cells transfection and microscopy methods

Cells were plated on coverslips and cultured in 35 mm diameter dishes for 2 days, reaching approximately 80% confluency. Plasmid DNA for transfection was prepared using an EndoFree plasmid isolation kit. 1 μg of plasmid DNA was used for each transfection. Transfection was performed using TransFast reagent according to the manufacturer's instructions. A 3:1 TransFast:DNA ratio was used, giving reasonable efficiency (approximately 20%) and low cytotoxicity. Two days after transfection, cells were washed with standard buffer and viewed under a Zeiss Axiovert microscope using a digital video imaging system equipped with a CCD Micromax 1300 YHS camera (Princeton Instruments) and MetaMorph software. A 480 nM fluorescence filter and ×100 oil-immersion objective were used. Z-scans of the cells were taken before and 5 min after agonist addition. Simultaneously, calcium measurement was performed with MetaFluor software in the subjected cells using Fura-2 imaging as described above, without conversion of the ratio values to $[Ca^{2+}]_i$. Z-scans were processed with AutoDeblur software using a blind deconvolution method.

2.8. Antisense oligonucleotide sequence and delivery

The antisense oligonucleotide sequence (5′-ACC TCG GTC ATC CTC-3′) was designed on the basis of the published sequence of $P2Y_1$ receptor (accession no. U22830). It covered 15 bp around the ATG start site of translation. The sense oligonucleotide was used as a control. Phosphothioate oligonucleotides were commercially synthesized and purified by high-performance liquid chromatography. For knockdown experiments, cells were grown on 35 mm diameter petri dishes (for cAMP assay) or on coverslips in 35 mm petri dishes (for intracellular calcium measurement) for 2 days reaching approximately 90% confluency. The oligonucleotides (5 μg per dish) were delivered in MEM without serum and antibiotics using Lipofectamine 2000 reagent according to the manufacturer's instructions. After 4 h, the medium was replaced by serum containing MEM and the cells were allowed to recover for 20 h. Then, the procedure was repeated. The cells were analyzed 24 h after the second transfection. The efficiency of this method of transfection reached 50%, as estimated with pS65T-C plasmid expressing GFP.

3. Results

The discovery that the PH domain of PLCδ binds PIP_2 with high affinity and selectivity makes this domain a powerful probe for determining the subcellular localization of this phospholipid [23]. In this study, we tested the binding specificity of the PH domain of PLCδ, expressed as a GFP fusion protein (PH(PLCδ) GFP) in glioma C6 cells. Digital fluorescence microscopy of PH(PLCδ) GFP-transfected glioma C6 cells revealed that the expressed protein was found within the cell. As shown in Fig. 1A, a distinct amount of fusion protein was constitutively present at the plasma membrane, indicating that, in unstimulated cells, PIP_2 resides in this compartment. Stimulation with ADP caused a rise in the intracellular calcium level (Fig. 1B). Simultaneously with the increase in $[Ca^{2+}]_i$, a loss of PIP_2 bound GFP fluorescence from the plasma membrane was observed. Fig. 1C demonstrates that, 5 min after stimulation of the same cell, PIP_2 bound GFP fluorescence was localized in the cytoplasm with no PIP_2 specific binding at the plasma membrane. This indicates that ADP-stimulated PIP_2 breakdown occurs in glioma C6 cells.

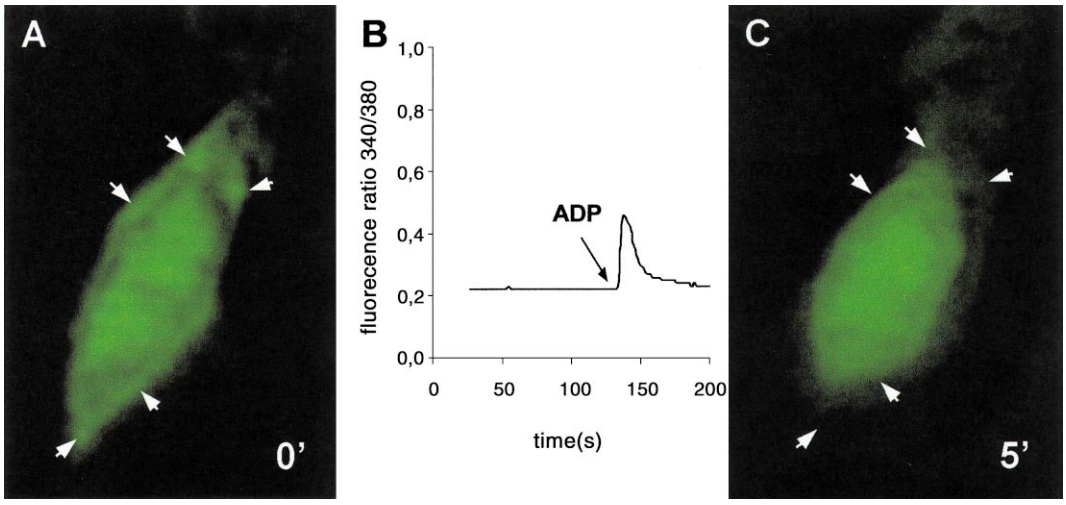

Fig. 1. ADP-stimulated PIP_2 breakdown in glioma C6 cells. Digital micrographs are shown of the same cell before (A) and after (C) 5 min of stimulation. The image shows the PIP_2 bound GFP distribution. Arrows mark points in the images that are described in the text. B: Calcium signal evoked by 10 μM ADP in the subjected cell. The data are representative of three independent experiments.

To differentiate whether ADP acts on one or two distinct P2Y receptors in these cells, we performed knock-down of $P2Y_1$ receptor in C6 glioma cells with antisense oligonucleotides [24]. To confirm the effects of the protocol used, RT-PCR was employed. Fig. 2 shows that, in the cells treated with antisense oligonucleotides, the mRNA expression level of $P2Y_1$ receptor was very strongly reduced (lane 4). The mRNA expression can be compared with control cells, untreated with antisense oligonucleotides (lane 3), or treated with sense oligonucleotides (lane 5), where the $P2Y_1$ mRNA was fully expressed. As an additional control the glyceraldehyde 3-phosphate dehydrogenase expression level was tested after transfection with antisense and sense oligonucleotides and found to be unchanged under these conditions (not shown).

In the next set of experiments, we examined the effect of knock-down of $P2Y_1$ receptor on ADP-induced Ca^{2+} mobilization and the inhibition of the isoproterenol-elicited cAMP accumulation (Fig. 3). The response to ADP in cells untreated with antisense oligonucleotides was taken as 100% and regarded as the control (Fig. 3). This ADP response consisted of both an increase in $[Ca^{2+}]_i$ level measured by digital fluorescence microscopy and the inhibition of the level of cAMP. As an additional control, we used the treatment of cells with sense oligonucleotides, which showed the same response to ADP as that observed in untreated cells. Fig. 3 shows that PPADS, the antagonist of PLC-coupled $P2Y_1$ receptor [14], strongly inhibited the ADP-evoked $[Ca^{2+}]_i$ increase. A similar effect was observed with MRS2179, a structurally related nucleotide. MRS2179, the specific and selective antagonist of

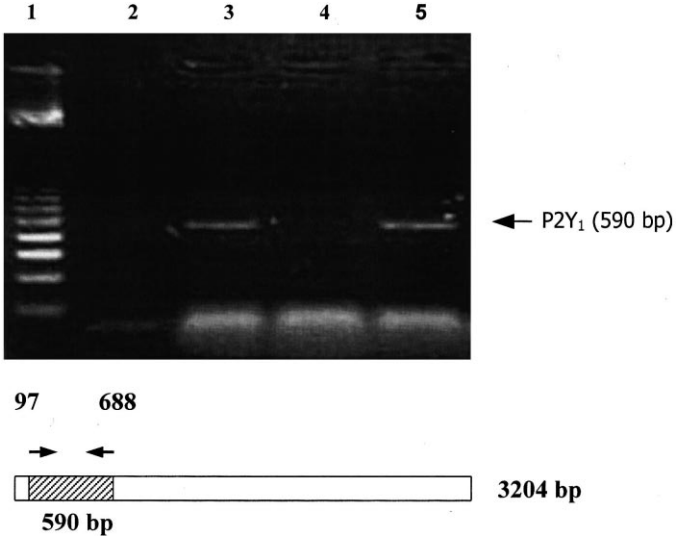

Fig. 2. Antisense oligonucleotides induced a decrease in $P2Y_1$ mRNA level. $P2Y_1$ expression in control and treated cells was compared by RT-PCR reaction. A 590 bp amplification product (corresponding to $P2Y_1$ mRNA) was analyzed on 1% agarose gel and visualized by ethidium bromide staining (for details see Section 2). Lane 1, molecular weight marker 123 bp; lane 2, control reaction in the absence of cDNA template; lane 3, control cells; lane 4, cells treated with antisense oligonucleotide; lane 5, cells treated with control sense oligonucleotide. All the reaction mixtures contained the same levels of template.

$P2Y_1$ receptor [25], completely blocked the ADP effect on the Ca^{2+} increase. In contrast, PPADS and MRS2179 did not abolish the ADP-induced cAMP inhibition. Knock-down of C6 cells with $P2Y_1$ antisense nucleotides markedly decreased (about 50% of the control, consistent with the efficiency of this method, see Section 2.8) the ADP-induced intracellular Ca^{2+} mobilization. In contrast, the ADP-evoked inhibition of adenylyl cyclase was unchanged, indicating, together with the effects of antagonists of $P2Y_1$ receptor, that two distinct ADP-responding receptors coexist in glioma C6 cells.

To confirm the identity of the $P2Y_1$-like receptor responsible for the inhibition of adenylyl cyclase in glioma C6 cells, PCR on reverse-transcribed total mRNA was employed. Primers were selected to cover the unique sequence of rat platelets $P2Y_{12}$ receptor. The estimated product size was 489 bp. Fig. 4 shows that mRNA of this receptor is expressed in glioma C6 cells.

4. Discussion

It is now generally accepted that in non-excitable cells, such as glioma C6 [18], biphasic capacitative Ca^{2+} signaling is mediated by the inositol system [26,27]. We have previously characterized $P2Y_1$ and $P2Y_2$ receptors in glioma C6 cells using measurements of $[Ca^{2+}]_i$ and demonstrated that ADP, ATP and UTP initiated a large Ca^{2+} response consistent with the typical capacitative model of Ca^{2+} entry, associated with

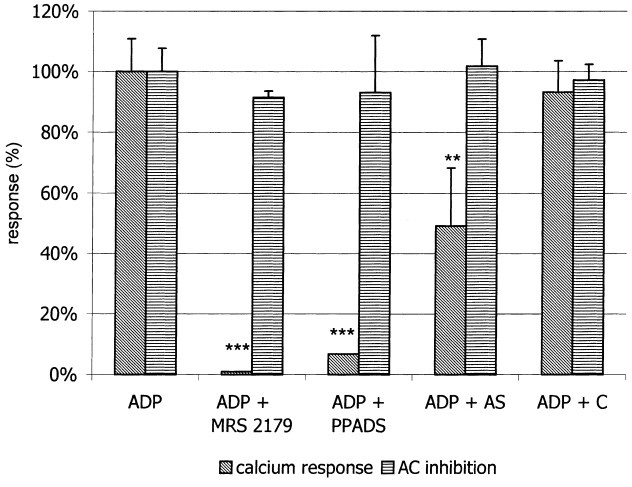

Fig. 3. Comparison of the effect of MRS2179, PPADS and antisense oligonucleotide treatment on ADP-induced Ca^{2+} mobilization and the inhibition of isoproterenol-elicited cAMP response. For the adenylyl cyclase assay, cells were treated with 50 μM isoproterenol in the absence or presence of ADP (10 μM) for 5 min at 37°C in the standard buffer. cAMP levels were assayed with the [^{3}H]cAMP kit. The Ca^{2+} assay was performed as described in Section 2. In the experiments concerning adenylyl cyclase activity, the inhibition by ADP of isoproterenol-evoked cAMP accumulation was taken as 100%. In the experiments concerning $[Ca^{2+}]_i$ the percentage of cells responding to ADP in the standard manner [15] was taken as 100%. MRS2179 (30 μM) and PPADS (100 μM) were added 2 min before the experiments and together with the agonist. Antisense treatment was performed as described in Section 2. The data are means ± S.D. from four independent experiments performed in duplicate (cAMP accumulation) or means ± S.D. from 10 independent experiments, with about 50 cells analyzed in each experiment (Ca^{2+} response). Statistical significance of differences is: $***P < 0.001$, $**P < 0.01$ (Mann–Whitney test). AS, cells transfected with antisense oligonucleotide; C, cells transfected with control sense oligonucleotides.

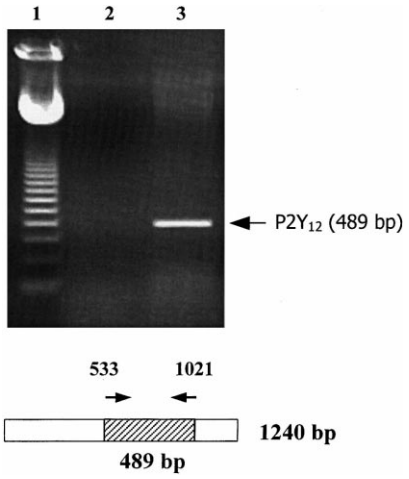

Fig. 4. Detection of $P2Y_{12}$ mRNA by RT-PCR. A 489 bp amplification product (corresponding to $P2Y_{12}$ mRNA) was analyzed on 1% agarose gel and visualized by ethidium bromide staining. Lane 1, molecular weight marker 123 bp; lane 2, control reaction in the absence of cDNA template; lane 3, PCR product for $P2Y_{12}$.

the stimulation of PLC and depletion of intracellular Ca^{2+} stores [15]. In this process, the initial rise in $[Ca^{2+}]_i$ results from the direct effect of $InsP_3$ on the endoplasmic reticulum and can be observed even in the absence of extracellular Ca^{2+} [28]. Our previous study showed that such Ca^{2+} responses were observed for all three nucleotides [15]. However, in contrast to ATP and UTP, the ADP-induced Ca^{2+} response was transient and quickly reduced to the basal level. Thus, one could expect that ADP-evoked $InsP_3$ accumulation was also transient and too short to be estimated. Such a short Ca^{2+} response was, most probably, the reason for the absence of a detectable increase in $InsP_3$ level in these cells and a former belief that, in glioma C6, ADP-responding nucleotide receptor stimulation does not lead to PLC activation [9,11,14]. Recently, Grobben et al. [29] demonstrated the increase in the $InsP_3$ level after ADP stimulation for 30 min. However, this increase was clearly seen at high concentrations of ADP, while ADP at low concentration was still able to produce a transient Ca^{2+} response. The authors explained this observation by PLC-independent Ca^{2+} influx, evoked by $P2Y_1$-like receptor ($P2Y_{AC}$, recently classified as $P2Y_{12}$).

The results of our previous study [15], and those presented herein, strongly indicate that, in C6 glioma cells, ADP evokes Ca^{2+} response by activating the $P2Y_1$ receptor, pharmacologically and molecularly identical to the cloned rat $P2Y_1$ receptor coupled to PLC. Therefore, we have concluded that in these cells $P2Y_1$ receptor stimulates PLC activation and is effective in the production of $InsP_3$ and release of Ca^{2+} from the endoplasmic reticulum stores. This conclusion was proved by the direct visualization of PIP_2 bound GFP fluorescence. After the stimulation with ADP, the PIP_2 bound GFP fluorescence at the cell surface was distinctly reduced with a simultaneous increase in $[Ca^{2+}]_i$. These results show the breakdown of PIP_2 by activated PLC. It is worth adding that no increase in the activity of phosphoinositide 3 kinase, another enzyme utilizing PIP_2 as a substrate, was observed during the time of the experiment (not shown).

Knock-down of $P2Y_1$ receptor with $P2Y_1$ antisense oligonucleotides markedly decreased the $P2Y_1$ mRNA level and evidently attenuated ADP-evoked intracellular Ca^{2+} mobiliza-

tion, whereas the ADP effect on adenylyl cyclase activity was unchanged. Similarly, the specific and selective $P2Y_1$ receptor antagonists, PPADS [14] and MRS2179 [25], completely blocked the ADP-evoked increase in $[Ca^{2+}]_i$ and did not change the cAMP level. These data elucidate the question concerning the presence of one (coupled to different G proteins) or two ADP-responding receptors in glioma C6 cells and clearly indicate the latter case: one coupled to PLC and elevated cytosolic Ca^{2+} and the other responsible for adenylyl cyclase down regulation.

To determine whether the ADP receptor coupled to adenylyl cyclase in glioma C6 cells is the same as that recently cloned from rat platelets and designated $P2Y_{12}$ [16], we investigated the expression of this receptor in C6 cells. RT-PCR analysis revealed that $P2Y_{12}$ is localized to these cells. The presence of this receptor was also reported in glioma C6-2B cells [17]. In platelets [16], C6-2B [17] and glioma C6 cells [15] this subtype of receptor is sensitive to PTX, indicating that it is coupled to adenylyl cyclase through G_i protein.

In conclusion, our data demonstrate that $P2Y_1$ and $P2Y_{12}$ receptor mRNAs are expressed in glioma C6 cells. ADP acts on these two receptors: $P2Y_1$, linked to the stimulation of PLC and Ca^{2+} release, and $P2Y_{12}$, negatively coupled to adenylyl cyclase through G_i protein.

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