

P2Y₁ nucleotide receptor silencing and its effect on glioma C6 calcium signaling

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In our earlier studies of the signaling cross-talk between nucleotide receptors in an *in vitro* glioma model (C6 cell line) under prolonged serum deprivation conditions, a growth arrest of the cells and expression shift from P2Y₁ to P2Y₁₂ receptors was found. The aim of the present work was to test if siRNA silencing of P2Y₁ receptor changes P2Y₁₂ expression similarly as following the serum deprivation and which physiological downstream pathways it affects. Here we demonstrate for the first time the efficiency of siRNA technology in silencing P2Y nucleotide receptors in glioma C6 cell line. Moreover, P2Y₁₂ proved to be insensitive to the P2Y₁ receptor silencing. The effect of the P2Y₁ silencing on calcium signaling was less pronounced than the extent of the protein change itself, exactly as was the case for the serum starvation experiments. Phosphorylation of ERK and Akt kinases were studied as the downstream effect of P2Y₁-evoked signaling and similar effects as in the case of serum deprivation were found for ERK, and even stronger ones for Akt phosphorylation.

Key words: siRNA, P2Y receptors, nucleotide receptors, calcium signaling

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INTRODUCTION

Signaling between the cell environment and the cytoplasm is fundamental for the ability of life to adapt to the changing conditions as well as for the regulation of the behavior of individual cells by the organism. In the cytoplasm, the signal is transmitted by so called secondary messengers, among which calcium ions are one of the most ubiquitous and versatile ones (Rasmussen *et al.*, 1976).

An important class of cell surface receptors capable of evoking calcium signals in the cytoplasm is a heterogenic group of receptors known as nucleotide receptors (Burnstock, 1976; Abbracchio & Burnstock, 1998; Braganhol *et al.*, 2009). These plasma membrane proteins are activated by extracellular nucleotides: ATP, UTP and their derivatives (ADP, UDP, UDP-glucose, UDP-galactose) (Burnstock, 2007). The nucleotide receptors are of two main types: P2X and P2Y. P2X receptors (P2X₁₋₇) belong to ligand-gated cation channels that after activation by ATP are able to form calcium transients (North, 2002). Glioma C6 cells (see below) *in vitro* express P2RX2, P2RX4, P2RX5 (Braganhol, *et al.*, 2009) whereas P2RX7 expression is a matter of debate (De Vuyst *et al.*, 2006; Wei *et al.*, 2008; Braganhol, *et al.*, 2009). Recently one of us proved that even though the P2X₇ protein is present in

these cells, the receptor is inactive (Suplat-Wypych *et al.*, 2010). Seven metabotropic G protein-coupled P2Y receptors are known so far: P2Y_{1,2,4,6,11,12,14} (Harden *et al.*, 2010). All of them respond to several agonists: purines (P2Y_{1,2,11,12}) as well as pyrimidines (P2Y_{2,4,6,14}) and sometimes several P2Y receptors share the same ligand. Such example of the common ligand is ADP or its non-hydrolysable analog 2-methylthio-adenosine-5'-diphosphate (2MeSADP), which activate both P2Y₁ and P2Y₁₂ receptors. However, the result of stimulation of these receptors is different as P2Y₁ is coupled to G protein containing G_q subunit G_q and P2Y₁₂ — G_i. Thus, in response to ADP or 2MeSADP action either calcium signaling or inhibition of adenylate cyclase occurs, respectively.

The rat glioma C6 cell line was used in this work as an *in vitro* model of primary glial tumor cells. The cell line belongs to a non-excitabile cell group (Baranska *et al.*, 1995) and is derived from chemically-induced rat brain tumor (Benda *et al.*, 1968). Glioma C6 shares several properties of astrocytic as well as oligodendrocytic progenitors and is often used as a biochemical model for studies related to astrocytes (Brismar, 1995). When injected into rodent brain these cells become morphologically similar to *glioblastoma multiforme* (Auer *et al.*, 1981) and therefore they are used in both *in vivo* and *in vitro* studies of this kind of tumor (Jacobs *et al.*, 2011). The expression and activity of several P2Y receptors: P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₂, P2Y₁₃ and P2Y₁₄ in glioma C6 has been well-documented in the literature (for review see: Wypych & Baranska, 2013).

In the past, several studies performed in Jolanta Baranska's laboratory, in which the authors of the present paper also participated, showed interesting crosstalk between G_q- and G_i-dependent pathways in the physiology of glioma C6 cells. This phenomenon was revealed by long-term serum starvation which affected the level of both P2Y₁ and P2Y₁₂ receptors protein. While the P2Y₁ protein level as well as the calcium signal evoked by its activation decreased during serum starvation, the level of P2Y₁₂ receptor rose (Krzeminski *et al.*, 2007). Those changes were interpreted as a shift from proliferative to pro-survival behavior under the unfavorable conditions of long-time serum deprivation.

The calcium signal evoked by G_q-coupled receptors, including P2Y nucleotide receptors, consists of two components and is preceded by activation of phospho-

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Abbreviations: 2MeSADP, 2-methylthio-adenosine-5'-diphosphate (IUPAC name: [(2R,3S,4R,5R)-5-(6-amino-2-methylsulfanylpurine-9-yl)-3,4-dihydroxyoxolan-2-yl]methyl phosphono hydrogen phosphate); CCE, calcium capacitative entry; TIRF, total internal reflection microscopy.

lipase C β and production of IP $_3$ followed by IP $_3$ action on its receptor (IP $_3$ R) on the endoplasmic reticulum (ER). This in turn leads to calcium release from ER cisternae (intracellular calcium stores) *via* IP $_3$ R. In SOCE (Store Operated Calcium Entry) the emptying of the ER cisternae is followed by opening of a special class of channels in the plasma membrane, so called store operated calcium channels (SOCs), and calcium flows from the cell environment into the cytoplasm. This influx is also known as capacitative calcium entry. Thus, there are two sources of calcium signal: intracellular calcium stores and the extracellular space.

A study of calcium signaling of serum-deprived glioma C6 cells revealed that the strength of the P2Y $_1$ receptor evoked calcium signal did not always correlate with the receptor protein level, but the observed phenomenon was explained by a compensating activity of P2Y $_{12}$ receptor (Suplat *et al.*, 2007).

Up to now there are no literature data about the regulation of transcription of *P2RY1* and *P2RY12* genes. To test the hypothesis that a decreased P2Y $_1$ expression enhances the expression of P2Y $_{12}$ receptor, we decided to silence the P2Y $_1$ receptor by siRNA and to test the effect of that on P2Y $_{12}$ protein level and the ability of the cells for nucleotide-dependent calcium signaling. The RNAi technology has proven to be an efficient tool to suppress the expression of targeted genes and, although never used earlier to target P2Y nucleotide receptors in glioma cells, it proved to be quite efficient in knocking-down P2X $_7$ receptor (Deli *et al.*, 2007).

MATERIALS AND METHODS

Cell culture. Rat glioma C6 cells were obtained from the American Type Culture Collection and cultured in DMEM (Invitrogen Life Technologies, USA) supplemented with 10% (v/v) newborn calf serum (NCS) (Invitrogen), penicillin (50 IU/ml) and streptomycin (50 μ g/ml) (Sigma-Aldrich, Germany) in a humidified atmosphere of 5% CO $_2$ at 37°C. For serum-starvation experiments, the medium was changed to DMEM without serum for up to 96 h (Czajkowski *et al.*, 2004; Krzeminski, *et al.*, 2007; Suplat, *et al.*, 2007).

P2Y $_1$ receptors silencing with siRNA. In all experiments a set of four dsRNAs selected for four regions of rat *P2RY1* mRNA (RefSeq NM_012800) was used (Dharmacon, Thermo Scientific, USA). Their sequences are shown in Table 1. Concentrations of siRNA were determined at 260 nm.

Glioma C6 transfection. Automatic electroporation system Amaxa (Lonza, USA) was used for cell transfection and only passages under 30 were used. Briefly, after non-enzymatic detachment (Cell Dissociation Solution, Sigma-Aldrich, Germany) 1.5 \times 10 6 cells were used for one reaction. After centrifugation (200 \times g for 10 minutes) the cells were resuspended in 100 μ l of electroporation buffer (Cell Line Nucleofector Solution V, Lonza, USA). Cells were electroporated using a survival-promoting

program in a Nucleofector II electroporator (Lonza, USA). For each electroporation from 0.25 to 3 μ g of siRNA was used. Immediately after electroporation cells were conditioned in 500 μ l of fresh culture media and then cultured appropriately for further experiments. As a positive control of electroporation, 2.5 μ g of GFP-coding plasmid was used (pmaxGFPTM, Lonza, USA). As negative controls, cells were either incubated in the presence of 3 μ g siRNA without electroporation or electroporated in electroporation buffer with no siRNA. The next day, transfection efficiency was calculated as a ratio between the number of GFP-expressing cells and all visible cells observed in at least 5 fields. A Nikon Diaphot microscope equipped with a 10 \times 0.5NA dry LWD lens was used using overlay of fluorescence and DIC Nomarski and the images were registered with a Retiga 1300 cooled digital camera (QImaging, Canada) controlled by the μ Manager program (Edelstein *et al.*, 2010).

Immunodetection of proteins. three days after electroporation cells were lysed in 100 μ l of RIPA buffer containing 150 mM NaCl, 1.0% IGEPAL $^{\text{®}}$ CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 (Sigma-Aldrich, Germany) with a cocktail of protease and phosphatase inhibitors, (Roche, Germany), and centrifuged for 10 min at 10000 \times g at 4°C. After determination of protein concentration with Bradford reagent (Bio-Rad), 20 μ g of protein was suspended in Laemmli buffer (50 mM Tris/HCl at pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol and 5% β -mercaptoethanol) and denatured at 98°C for 5 min. For gel electrophoresis 10% polyacrylamide gel was used. Proteins were then transferred to a nitrocellulose filter (0.45 μ m, Bio-Rad, Germany) by wet transfer in a buffer containing 25 mM Tris/HCl, 192 mM glycine, 0.1% SDS, 10% methanol for 60 min at 400 mA in a cold room. Transfer efficiency was controlled by staining in Ponceau S solution (Sigma Aldrich, Germany).

Nitrocellulose membranes were incubated at room temperature for 60 minutes in blocking buffer composed of 5% w/v nonfat dry milk in PBS with 0.05% Tween 20 (PBS-T) and then incubated at 4°C overnight in blocking buffer containing primary antibodies. The following dilutions of primary antibodies were used: 1:1000 anti-P2Y $_1$ rabbit, 1:200 anti-P2Y $_{12}$ rabbit (both from Alomone Labs, Israel), 1:1000 both anti-phospho p-42/44 MAPK (Erk1/2) and anti-phospho-Akt (Cell Signaling, USA); for housekeeping control, 1:50000 monoclonal anti- β -actin conjugated with horseradish peroxidase (Sigma-Aldrich, Germany) was used. After four 10-minute washes in PBS-T, the blots were incubated for 1 h with anti-rabbit secondary antibody (1:5000) conjugated with horseradish peroxidase (Pierce, USA). West Femto Maximum Sensitivity Substrate (Pierce, USA) was used to detect horseradish peroxidase activity and evaluate the amount of protein.

To evaluate changes in the amounts of P2Y $_1$ and P2Y $_{12}$ receptors, membranes were first detected for P2Y $_1$ followed by β -actin and then for P2Y $_{12}$ with stripping between different antibodies. The same procedure was used for phospho-Erk and phospho-Akt. Stripping buffer contained 0.2 M glycine, 0.1% SDS and 0.01% Tween 20. After two rounds of stripping (7 minutes each), membranes were washed twice for 10 minutes in PBS and twice for 5 minutes in PBS-T, followed by blocking and hybridization as above. The specificity of the anti-P2Y $_1$ and anti-P2Y $_{12}$ antibodies was confirmed as in (Krzeminski, *et al.*,

Table 1. dsRNA sequences used for silencing P2Y $_1$ receptor.

	Sense	Antisense
1	UGGCACUGGGAUUCGAAAUU	5'-P UUUCCGAAUCCAGUGCCAUU
2	GAUGAGUACCUGCGAAGUUUU	5'-P AACUUCGCGGUACUCAUUU
3	GGAUUAUUUGUUAGAGCUUUU	5'-P AAGCUCUAACAAUUAUCCUU
4	CGGUGUACAUGUCAAUUUUU	5'-P AAUUGAACAUUGUACACCGUU

2007) by incubation of the primary antibodies with antigen peptide provided by the manufacturer. A 1:1 antigen to antibody mixture was pre-incubated for 1 h (with agitation) in blocking buffer and then membrane was processed as for primary antibody.

Multiple bands visible on all P2Y₁ blots are common problem for most P2Y receptor detection experiment. As discussed earlier, it is not nonspecific staining but the result of several glycosylation forms of the same receptor present in the cell. Decylosylation of cell extract by N-glycosidase F results in one band on the western blot (Krzeminski *et al.*, 2008).

Analysis of the immunodetected proteins. Western blots were imaged on X-Ray film (Kodak), scanned and then densitometry was performed with ImageJ program plugin (Schneider *et al.*, 2012). Signal intensity was normalized first to β -actin than to that in control conditions and the values shown are mean of at least three experiments \pm SD. In order to determine statistical significance of differences nonparametric Mann-Whitney U test was used. The significance is labeled (*) for $p < 0.05$, (***) for $p < 0.001$ and a lack of statistical significance is presented as (-).

Immunostaining of P2Y₁ receptors for wide field and TIRF microscopy. Cells were grown on TIRF-compatible, high precision N° 1.5 coverslips (Paul Marienfeld GmbH&Co.KG, Germany). After washing twice with PBS, cells were fixed in cold (4°C) acetone for 3 min, rehydrated in PBS for at least 10 minutes at RT and then permeabilized for 10 minutes with 0.1% Triton X100 (Romil Chemicals, UK). To confirm specificity of the staining, primary anti-P2Y₁ antibody was preincubated with the peptide provided by the manufacturer at a 1:1 dilution, in the same way as in the case of western blot. After careful washing, slides were incubated in 2.5% NCS in BPS (ICC blocking solution) for 1 hour to block unspecific binding and then stained overnight (4°C) with primary anti-P2Y₁ antibody (Alomone, Israel) diluted 1:200 in ICC blocking solution. After three 10-minute washes in PBS slides were incubated with secondary goat anti-rabbit IgG antibodies conjugated with Alexa488 (Invitrogen, Life Technologies, USA) in ICC blocking solution (1:600) for 2 hours at RT. After three 10-minute final washes in PBS, specimens were mounted with VectaShield mounting medium (Vector Laboratories, UK). For evaluation of P2Y₁ receptor staining a Carl Zeiss Laser TIRF3 system was used. It contained a Carl Zeiss Axio Observer Z.1 microscope stand (Carl Zeiss, Germany) equipped with a 488 nm/100 mW argon laser for TIRF and a mercury arc lamp for wide field fluorescence microscopy. High numerical aperture plan-apochromat lens 100 \times 1.46NA was used to provide total reflection of side introduced laser beam in the coverslip and allow the TIRF mode. Images were collected with a high efficiency back-thinned CCD camera Quantem 512SC (Photometrics, Germany) for n cells from at least 5 different areas. The microscope was controlled by AxioVision 4.7.2.0 software (Carl Zeiss, Germany). Ratio between images of background-corrected TIRF and wide field fluorescence images was calculated with MetaMorph 7.5.6.0 software (Danaher Corporation, USA). In graphs, the ratio TIRF/WF in control conditions is presented as 1 and relative changes (mean \pm S.E.) of the parameter are shown. The imaging in each experimental condition was performed at least three times.

Calcium imaging. The experiments were performed as described before (Suplat-Wypych, *et al.*, 2010). Briefly, before experiments cells on coverslips were washed once with PBS and once with solution containing 137 mM

NaCl, 2.7 mM KCl, 1 mM Na₂HPO₄, 25 mM glucose, 20 mM Hepes (pH 7.4), 1 mM MgCl₂, 1% bovine serum albumin and 2 mM CaCl₂ (standard buffer). The cells were then incubated at 37°C for 30 min in the standard buffer with 1 μ M Fura-2 AM. Thereafter, the coverslips were mounted in a chamber on a Nikon Diaphot inverted-stage microscope equipped with a fluo \times 40/1.3 NA oil-immersion objective lens. Fura-2 digital fluorescence microscopy was used to determine changes in the intracellular calcium levels [Ca²⁺]_i (Grynkiewicz *et al.*, 1985).

Ludl Lep MAC 5000 filter wheel system with a Chroma Fura-2 filter set was used for illumination of specimens. Images were acquired using an Andor Luca R digital EMCCD camera (Andor, Irland). Data processing was carried out using iQ Live Cell Imaging Software (Andor, Irland) and Matlab® software (The Mathworks Inc, USA). All data are expressed as 340/380 nm-induced fluorescence of Fura-2 ratio changes against time (Δ 340/380). Each experiment was repeated at least three times. MeSADP (Sigma-Aldrich, Germany) at 10 μ M was used to evoke P2Y₁-dependent calcium signal. Calcium responses against time are expressed as means of (n) cells. Changes in the calcium response were calculated as the relative changes of integrals from 100 consecutive measuring points after adding the agonist (mean \pm S.D.).

RESULTS

The receptor protein level during serum deprivation

Previously published serum starvation experiments (Krzeminski *et al.*, 2007) were carried out in significantly different conditions. Change of the glioma C6 cell line passage from above 40 to below 30 was forced by electroporation protocol and lead to difference in obtained results large enough to repeat receptor level measurements as a reference for further research.

Using western blot analysis, we measured relative protein content of P2Y₁ and P2Y₁₂ receptors in glioma C6 cells during prolonged, 96 h serum deprivation as a starting point of the study (Fig. 1). The amount of P2Y₁ receptor protein fell to less than one third of control level (10–30%, depending on the experiment). At the same time the amount of P2Y₁₂ receptor protein rose to about 150% of the control. Although those changes were less pronounced than the ones reported previously (Krzeminski, *et al.*, 2007), the trends were exactly the same: the level of P2Y₁ receptor was falling, and of the P2Y₁₂ receptor rising and, crucially, in both series of measurements, the increase of P2Y₁₂ receptor level during prolonged serum starvation was three times smaller than decrease of P2Y₁ receptor level. In other words, the rise of P2Y₁₂ is evident, but less intense than the fall of P2Y₁ receptor. Experiments were performed four times, data pooled and processed together.

Silencing of P2Y₁ receptors by siRNA

Since we wanted to evaluate the effect of progressive P2Y₁ silencing, and not just a lack of P2Y₁ protein, the transient siRNA approach was chosen. The poor efficiency of other transfection methods (around 3% after lipofectamine treatment, not shown) made us use electroporation with the Amaxa system as an effective way to deliver small RNA molecules into cells. Preliminary experiment with GFP vector (pmaxGFP_{TM}, Lonza, USA) proved transfection efficiency above 40–70%. The best result of silencing we observed 48 h after electropo-

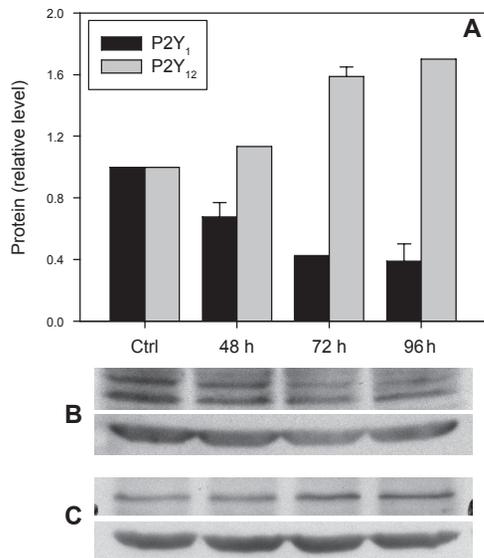


Figure 1. Effect of long-term starvation on P2Y₁ and P2Y₁₂ receptors protein level

(A) Densitometric analysis of western blot. Four independent experiments were performed. (B) Typical result of P2Y₁ receptor protein detection, here shown control, 48h, 72 h and 96h of serum-deprivation time points. Lower row: β -actin as housekeeping protein reference. (C) Typical example of P2Y₁₂ receptor protein detection, here shown control, 48 h, 72 h and 96 h time points of serum deprivation. Lower row: β -actin as housekeeping protein reference.

ration with siRNA. Figure 2 shows dose dependency of P2Y₁ receptor silencing on the amount of siRNA loaded into the cells. The siRNA dose of 0.25 μ g was ineffective (1.02 ± 0.15), and the effect was growing with the dose of siRNA (0.72 ± 0.47 for 0.5 μ g) up to 0.32 ± 0.07 for

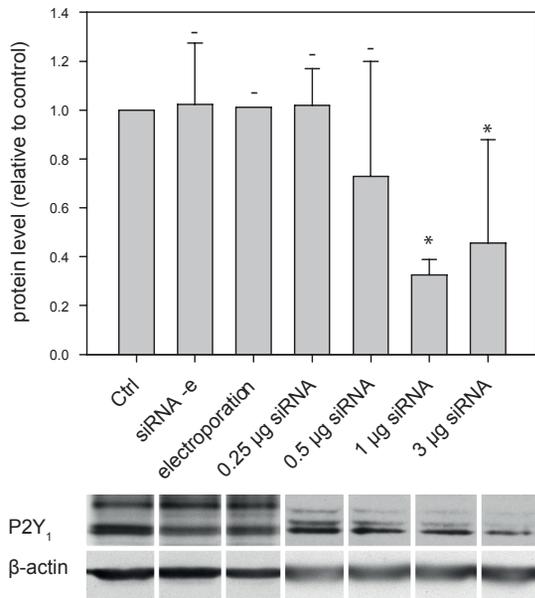


Figure 2. Silencing of P2Y₁ receptor by siRNA

P2Y₁ protein level two days after electroporation of glioma C6 cells with specific siRNA. Densitometric analysis of the western blot, from left: control, cells incubated with specific siRNA without electroporation, cells electroporated in the control medium, without siRNA, growing dose of siRNA: 0.25 μ g, 0.5 μ g, 1 μ g and 3 μ g. The dose-dependent activity of specific siRNA can be observed from 0.25 μ g to 1 μ g dose. Presented blots come from two representative experiments, β -actin as housekeeping protein reference.

1 μ g. With 3 μ g we observed erratic results (0.45 ± 0.42), showing that the effect may not be specific any longer. It could be due to a too high (higher than recommended by the producer) dilution of the sample by siRNA. The addition of 3 μ g of siRNA without electroporation or the electroporation without the siRNA in the medium did not result in any statistically significant changes of P2Y₁ receptor protein level in the cells (1.02 ± 0.25 and 1.01 , respectively). Altogether it shows that the observed results are specific consequences of the siRNA activity in glioma C6 cells, and are not the result of the experimental procedure itself. For further experiments we decided to use 1 μ g of siRNA.

The maximal level of silencing was observed after 48 h of silencing with 1 μ g siRNA (0.32 ± 0.07 of control) what may be caused by the slow turnover of the receptor in the idle cells. To find out if the protein was still present on the plasma membrane, we decided to use TIRF (total internal reflection fluorescence) microscopy (Fig. 3). TIRF microscopy allows one to observe a 100-nm layer of the specimen close to the coverglass. With this method we evaluated the amount of the P2Y₁ receptor protein present on the plasma membrane. In contrast, in wide field (WF) fluorescent microscopy the signal is proportional to the total amount of a protein in the cell. Thus, the ratio of fluorescence intensity in the TIRF channel to that in the wide field channel is proportional to the ratio between membrane and total pool of receptors. We measured the TIRF/WF signal ratio of immunocytochemically stained P2Y₁ receptors and found that 48 h after siRNA administration the amount of P2Y₁ receptors on the cell surface fell faster than did the total receptor protein level (calibrated ratio:

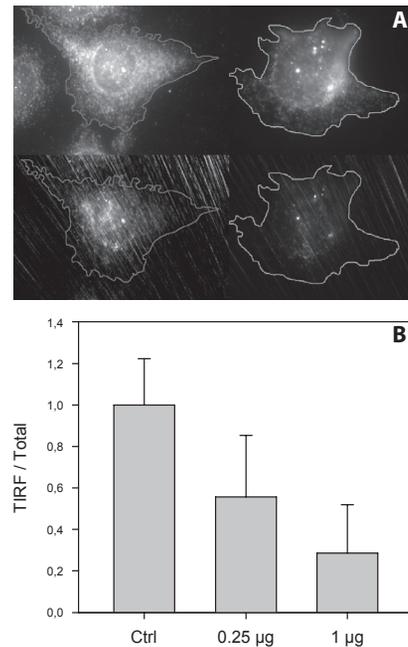


Figure 3. Distribution of P2Y₁ receptor after prolonged siRNA silencing

Glioma C6 cells were stained with anti-P2Y₁ antibody and visualized both in wide field (WF) and total internal reflection fluorescence (TIRF) microscopy. (A) (upper row) Fluorescence image of control C6 cell stained with antibody against P2Y₁ receptor. The same cell pictured in the TIRF mode (lower row). Left column: control cells, right column: cells with P2Y₁ receptors silenced by 1 μ g dose of siRNA. (B) Bars (mean \pm S.E.) present the ratio between TIRF and WF fluorescence intensity, proportional to the ratio between internalized and membrane fraction of receptor.

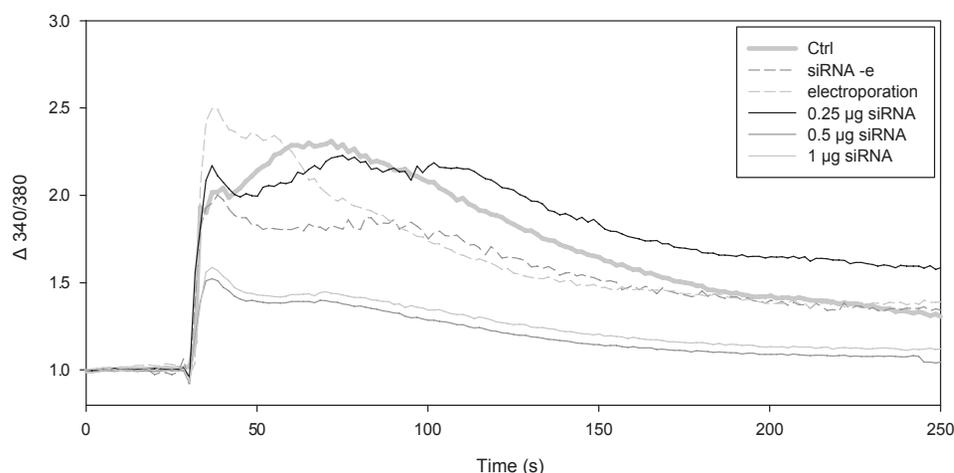


Figure 4. siRNA silencing of P2Y₁ receptor — effect on calcium signaling

Glioma C6 cells were loaded with Fura-2 AM and signal evoked by 10 μ M 2MeSADP in the presence of 2 mM CaCl₂ was studied. Thick grey plot shows control cells without any treatment ($n=267$). Dashed plots present calcium signal of the transfection control experiments: medium grey — cells incubated with 1 μ g siRNA but not electroporated ($n=282$) and light grey — cells electroporated in absence of siRNA ($n=412$). Note that calcium signal of the electroporated cells has stronger ER release phase and shorter capacitative phase. All plots present the mean response from n number of cells as indicated in brackets. Solid plots present calcium signal of the cells with P2Y₁ protein knock-down by siRNA: black — 0.25 μ g ($n=213$), medium grey — 0.5 μ g ($n=254$), light grey — 1 μ g ($n=187$). siRNA doses which earlier significantly lowered P2Y₁ receptor protein level also strongly inhibited calcium signal.

control 1.0 ± 0.01 , 0.5 μ g siRNA: 0.55 ± 0.014 and 1.0 μ g 0.29 ± 0.011 .

Physiological effect of P2Y₁ receptor silencing — calcium signaling

Knowing the effects of individual siRNA doses on the total P2Y₁ protein level and its level in the cell membrane, we could test the influence of both the experimental procedure and receptor knock-down on the direct signaling effect of its activation: the calcium signal in the cytoplasm. Evoked by 10 μ M MeSADP, it was studied with Fura-2 ratiometry, as described in Materials and Methods. Dashed lines in figure 4 present the transient plots in control experiments while solid ones in siRNA-treated cells. It worth mentioning, that electroporation of the cells without siRNA changes the shape of the calcium signal in comparison with control, even 48 hours after electroporation. The initial peak of the signal

(calcium release from ER) reached higher values and the second, CCE sustained phase, even if initially stronger, disappeared faster. A similar signal shape was seen in all siRNA-treated cells, with no CCE phase, suggesting some long-term influence of electroporation on cell membranes. To quantify the effect of P2Y₁ siRNA administration on the calcium signal strength, we decided to calculate integrals of the transients, to compare plots with slightly different shapes (Fig. 5). Contrary to the protein level effects, both electroporation and incubation with siRNA without electroporation led to small but statistically significant diminishing of the signal (0.75 ± 0.05 for cells incubated with siRNA and 0.87 ± 0.03 for cells electroporated in absence of siRNA, both as fraction of control signal). Surprisingly, we did not observe such an effect after electroporation with low dose (0.25 μ g) of siRNA (1.04 ± 0.04). The effect of the siRNA doses, which significantly changed the protein level was several times stronger. Surprisingly, we did not observe the dose-dependency as in the protein level (0.24 ± 0.03 for 0.5 μ g of siRNA and 0.27 ± 0.02 for 1 μ g of siRNA).

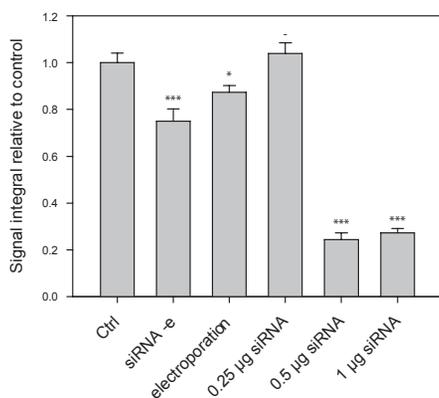


Figure 5. siRNA silencing of P2Y₁ receptor — quantification of calcium signaling

Signals presented in Fig. 4 were integrated. Both negative controls groups (cells incubated with 3 μ g siRNA without electroporation and electroporation in absence of siRNA) cause slight, but statistically significant inhibition of the calcium signal. Such an effect is not visible in cells electroporated with low (0.25 μ g) dose of siRNA. Higher doses (0.5 and 1 μ g) reduced signal very strongly, however the effect is not dose dependent.

Physiological effect of P2Y₁ receptor silencing — downstream effects

We also studied the level of phosphorylation of ERK1/2 (p44/p42) and Akt, well known targets of the calcium signal in glioma C6 cells. Phosphorylation of both proteins was studied up to 120 min after induction of cells by 10 μ M 2MeSADP (Fig. 6). Control cells (black bars) were compared with those electroporated with 1 μ g of siRNA silencing the P2Y₁ receptor (grey bars). While Erk1/2 phosphorylation was only partially (more than two times) diminished, Akt phosphorylation was practically blocked.

Effect of P2Y₁ receptor knock-down by siRNA on P2Y₁₂ receptor protein level

The main goal of this work was to find out if the increase of the P2Y₁₂ protein level observed during serum deprivation of C6 glioma cells was a simple compensatory effect of the P2Y₁ receptor level fall. To evalu-

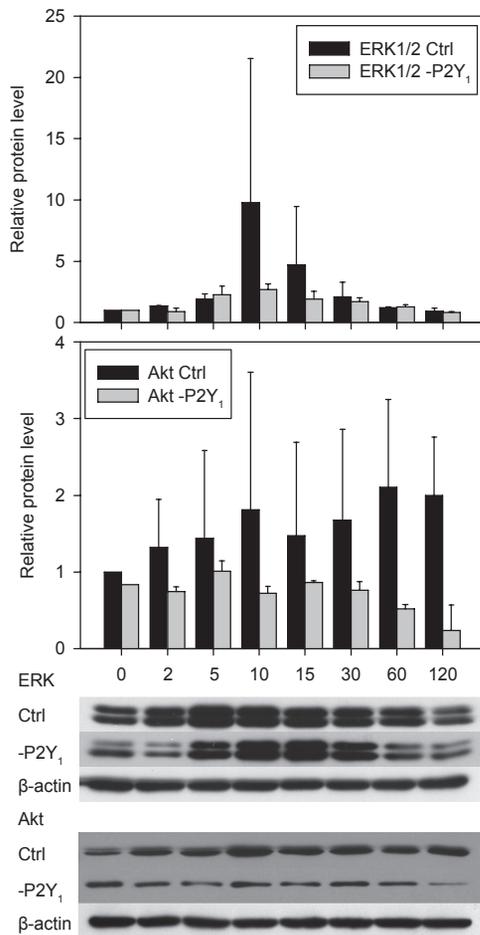


Figure 6. siRNA silencing of P2Y₁ receptor — effect on downstream signal

Phosphorylation of Erk1/2 kinase (A) and Akt (B) was studied in control cells and cells electroporated with 1 μ g siRNA in several time points after 10 μ M 2MeSADP stimulation. After silencing of P2Y₁ receptor (grey bars) the level of Erk1/2 phosphorylation is strongly attenuated in comparison with control cells (black). The Akt Phosphorylation is practically blocked by P2Y₁ receptor knock-down by siRNA. Representative blots together with β -actin as housekeeping protein reference shown.

ate this we analyzed P2Y₁₂ protein level in parallel with P2Y₁ during siRNA P2Y₁₂ silencing (Fig. 7). The amount of P2Y₁₂ reached 1.20 ± 0.17 for siRNA administration without electroporation, 1.16 for electroporation without siRNA, 1.03 ± 0.07 for 0.25 μ g siRNA, 0.92 ± 0.07 for 0.5 μ g siRNA, 1.02 ± 0.02 for 1.0 μ g siRNA, and 1.13 ± 0.11 for 3.0 μ g siRNA. There was no influence of the P2Y₁ protein depletion on the P2Y₁₂ receptor level. The observed differences were small and statistically insignificant.

DISCUSSION

This study clearly shows that siRNA silencing of P2Y nucleotide receptors is a promising experimental approach. The siRNA knock-out achieved in the present work was efficient but not complete. It is better than that reported by Deli *et al.* (2007) for the P2X₇ receptor in melanoma cells (about 50% of downregulation) and similar to the one recently reported by Crooke *et al.* (2009) and Martin-Gil *et al.* (2012) both on P2Y₂ receptor. This level of efficiency is probably a result of two

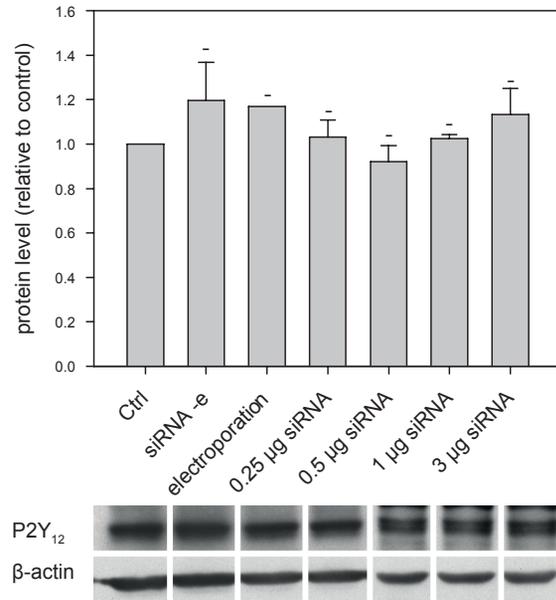


Figure 7. siRNA silencing of P2Y₁ receptor — effect on P2Y₁₂ receptor protein level

Glioma C6 cells content of P2Y₁₂ protein level after electroporation with 1 μ g of specific siRNA. Densitometric analysis of western blot of three independent experiments. From left: control, cells incubated with 3 μ g of siRNA without electroporation, cells electroporated in the electroporation buffer, without siRNA, growing dose of siRNA: 0.25 μ g, 0.5 μ g, 1 μ g and 3 μ g. As, shown in Fig. 1, the dose dependent activity of specific siRNA was observed on P2Y₁ protein level between 0.25 μ g and 1 μ g. There is no visible effect of this inhibition on the observed level of P2Y₁₂ protein level. Presented blots come from two representative experiments, β -actin as housekeeping protein reference.

causes: the siRNA delivery efficiency and the relatively slow receptor protein turnover. The latter possibility is supported also by our data from TIRF imaging, showing gradual transfer of the P2Y₁ receptors from ventral plasma membrane into the cytoplasm.

The observed physiological effects of inhibition of Phosphorylation of Erk1/2 and Akt kinases after silencing of P2Y₁ receptors follows the Sak and Illes postulate that a general property of neuronal malignant cells appears to be the absence of P2Y₁ receptor and inhibition of PI3-K/Akt signaling (Sak & Illes, 2005). In the present study the effect is much more pronounced than in the serum deprivation paper, where Akt signaling was strongly affected after long-term experiment (Krzeminski, *et al.*, 2007). Moreover, our previous work clearly shows that P2Y₁₂ receptors have a positive, modulatory influence (*via* cAMP-dependent pathways) on the P2Y₁-dependent calcium signaling (Suplat, *et al.*, 2007). However, the mechanism proposed by Hardy *et al.* (2004) for platelets does not seem to depend strongly on the P2Y₁₂ receptor protein level itself, even if the activation of this receptor strongly affects the cAMP level in glioma C6 cells *via* G_i protein (Van Kolen & Slegers 2004). Moreover, the P2Y₁-evoked calcium signal observed in the present work in glioma C6 cells was much less sensitive to the siRNA dose than the receptor protein level itself. Those discrepancies between the receptor protein level and the signaling efficiency can, however, have much deeper roots.

Several years ago it was discovered that another nucleotide receptor, P2Y₂, can interact with integrins, changing the signaling activity after entering into complex with $\alpha_v\beta_5$ integrins (Bagchi *et al.*, 2005) and changing its G protein α subunit specificity from G_i to G₁₂ (Liao *et al.*,

2007). This change switches the signal from the receptor and instead of inducing the calcium transient the receptor directly activates RhoA-dependent signaling pathway (Suzuki *et al.*, 2003). This quite unique double specificity of GPRS receptors to G_q and G_{12/13} subunits of heterotrimeric G proteins may lead to the situation in which the level of receptor protein does not exactly correlate with the signal from this receptor. Even if the ability to bind integrins was described only for ATP-induced P2Y₂ receptors, notions of integrin-dependent ADP nucleotide signaling appeared recently (O'Brien *et al.*, 2012) as well as the P2Y₁₂-dependent integrin activity influencing microglia cell spreading under the ADP stimulation (Ohsawa *et al.*, 2010).

Summarizing, this paper shows that nucleotide signaling is an interplay of two proteins more complicated than previously postulated and the cross-talk between receptors may be very indirect and finely modulated by the cell structure and function.

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