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ORIGINAL PAPER

$P2Y_1$ and $P2Y_{12}$ receptor cross-talk in calcium signalling: Evidence from nonstarved and long-term serum-deprived glioma C6 cells

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Abstract The current work presents results of experiments on the calcium response evoked by the stimulation by extracellular nucleotides occurring in control, nonstarved glioma C6 cells and in cells after long-term (96 h) serum starvation. Three nucleotide receptors were studied: $P2Y_1$, $P2Y_2$ and $P2Y_{12}$. Two of them, $P2Y_1$ and $P2Y_2$, directly stimulate calcium response. The protein level of the $P2Y_2$ receptor did not change during the serum starvation, while $P2Y_1$ protein level fell dramatically. Observed changes in the calcium response generated by $P2Y_1$ are directly correlated with the receptor protein level as well as with the amount of calcium present in the intracellular calcium stores, partially depleted during starvation process. The third receptor, $P2Y_{12}$, did not directly evoke calcium response, however it is activated by the same ligand as $P2Y_1$. The experiments with AR-C69941MX, the $P2Y_{12}$-specific antagonist, indicated that in control and serum-starved cells, calcium response evoked by $P2Y_1$ receptor is potentiated by the activity of $P2Y_{12}$-dependent signaling pathways. This potentiation may be mediated by $P2Y_{12}$ inhibitory effect on the plasma membrane calcium pump. The calcium influx enhanced by the cooperation of $P2Y_1$ and $P2Y_{12}$ receptor activity directly depends on the capacitative calcium entrance mechanism.

Key words AR-C69931MX · calcium signalling · capacitative calcium entry · Glioma C6 · nucleotide receptors · serum starvation

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Introduction

In most cell types, a large number of cell functions are regulated by the level of cytoplasmic Ca^{2+}. Efficient calcium signalling requires a Ca^{2+} source as well as mechanisms for Ca^{2+} depletion after a period of activation. Studies on the Ca^{2+} source have led to division of all cells into two types, excitable and nonexcitable, based on the presence of voltage-dependent calcium channels in their plasma membranes [1, 2]. Transformed glial cells, such as glioma C6, belong to the nonexcitable cells [3, 4]. We have reported that an increase in KCl concentration in the extracellular medium to 140 from 2.7 mM does not produce any change in the intracellular Ca^{2+} level in glioma C6 cells [3]. On the contrary, under such conditions, excitable cells open voltage-dependent Ca^{2+} channels via membrane depolarization, and massive influx of Ca^{2+} is observed [1, 2].

Nonexcitable cells are characterized by capacitative Ca^{2+} entry, also known as store-operated Ca^{2+} entry, regulated by the concentration of calcium ions in the endoplasmic reticulum (ER) pool [5–8]. The depletion of this pool (the first phase of calcium response) causes the opening of voltage-independent Ca^{2+} channels in the plasma membrane (PM), which permits the Ca^{2+} entry (second phase of calcium response). First messengers mediating inositol-1,4,5-trisphosphate (IP_3) formation cause release of Ca^{2+} from the ER via IP_3 receptors followed by Ca^{2+} entry into the cell as a consequence of the depletion of the ER store [5–8].

We have previously reported that, in glioma C6 cells, stimulation of nucleotide receptors by agonists ATP, UTP, ADP, or ADP-analogue 2-methylthioadenosine-5'-diphosphate (2MeSADP) initiates a biphasic Ca^{2+} response compatible with the typical capacitative model of Ca^{2+} influx [9–12]. Among the large family of metabotropic P2Y nucleotide receptors, $P2Y_2$ responds to ATP and UTP,

whereas $P2Y_1$ and $P2Y_{12}$ respond to ADP and 2MeSADP. $P2Y_1$ and $P2Y_2$ receptors are both coupled to phospholipase C (PLC) and are responsible for Ca^{2+} mobilization from intracellular stores, while the $P2Y_{12}$ receptor is negatively coupled to adenylate cyclase [13–17]. We have also shown that differences in the cell culture conditions, i.e. the presence or absence of serum in the cell culture medium, have a strong effect on $P2Y_1$ receptor mRNA expression, which strongly decreases in the absence of serum [9, 10].

This effect is so strong that Slegers group [18], growing glioma C6 cells in serum-free chemically-defined medium, could not evoke $P2Y_1$ receptor-dependent PI turnover and suggested that, in these cells, the $P2Y_{12}$ receptor was not only coupled to inhibition of adenyl cyclase but also to activation of PLC-independent Ca^{2+} influx. According to the authors, the mechanism of $P2Y_{12}$-dependent calcium influx remained to be determined, however, in Fig. 8 of [19] and Fig. 1 of [20], they proposed the interesting idea that stimulation of the $P2Y_{12}$ receptor might generate, via $G\beta\gamma$ subunits of G_i protein, a direct entry of extracellular calcium [19, 20].

The aim of the present study was to check whether the $P2Y_{12}$ receptor may be directly responsible for the calcium entry. Our results indicate a full correlation between the $P2Y_1$ receptor protein level and the extent of Ca^{2+} response in glioma C6 cells. Moreover, although Ca^{2+} response evoked by the $P2Y_1$ receptor may be potentiated by $P2Y_{12}$-dependent signalling pathways, the hypothesis proposed by other authors [18–20], that in this cell line $P2Y_{12}$ is directly responsible for Ca^{2+} influx, seems to be doubtful. Thus, data presented herein clarify the problem of the roles that both receptors play in the calcium response.

Materials and methods

Materials

Dulbbeco's Modified Eagle's Medium (DMEM) and newborn calf serum (NCS) were obtained from Gibco BRL. ADP, 2MeSADP, UTP, EGTA, phosphate-buffered solutions (PBS), penicillin and streptomycin were purchased from Sigma Aldrich Chemical. Fura-2/AM was from Molecular Probes. AR-C69931MX was a kind gift from AstraZeneca (Wilmington, DE, USA). MRS 2179 tetraammonium salt was from Tocris. Antibodies recognizing $P2Y_1$, $P2Y_2$, and $P2Y_{12}$ were purchased from Alomone and Sigma Aldrich Chemical labs. Horseradish peroxidase-conjugated anti-rabbit IgG was from Cell Signalling. Nitrocellulose membrane and enhanced chemiluminescence detection system (ECL) were from Amersham Pharmacia Biotech. Medical X-ray films were from Foton Trading Poland. All other reagents were purchased from Sigma Chemical.

Cell culture

Rat glioma C6 cells (passages 40–60) were obtained from American Tissue Culture Collection and cultured in DMEM with high glucose (4,500 g/dm^3) and GlutaMAX I, supplemented with 10% (v/v) NCS, penicillin (100 UI/ml) and streptomycin (50 μg/ml) under humidified atmosphere of 5% CO_2 at 37°C. For experiments, control cells were cultivated in DMEM supplemented with 10% NCS to reach 90% confluence in 60-mm dishes (for Western blot analysis) or on 22-mm glass coverslips in 35-mm dishes (for calcium measurement). In case of serum-starved cells, the medium was changed to DMEM without NCS 96 h before the experiment.

Measurement of intracellular calcium

Thirty minutes before the calcium measurements, cells on coverslips were washed once with PBS and once with solution containing 137 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO_4, 25 mM glucose, 20 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 1% bovine serum albumin and 2 mM $CaCl_2$ (standard buffer). In experiments performed in the absence of external Ca^{2+}, 500 μM EGTA was added instead of 2 mM $CaCl_2$. The cells were then incubated at 37°C for 30 min in the standard buffer with 2 μM Fura-2 AM. Thereafter, the coverslips were mounted in a chamber on a Nikon Diaphot inverted-stage microscope equipped with a fluo ×40/1.3 NA oil-immersion objective lens. Fura-2 digital fluorescence microscopy was used to determine the changes in intracellular calcium levels $\left([Ca^{2+}]_i\right)$ [21].

Ludl Lep MAC 5000 filter wheel system loaded with a Chroma Fura-2 filter set was used for illumination of specimens. Images were acquired using Retiga 1300 chilled digital CCD camera (QImaging). Data processing was carried out using AQM Advance 6 (Kinetic Imaging Inc) and MS Excel software. All data are expressed as 340/380 nm-induced fluorescence of Fura-2 ratio changes against time (Δ340/380). Each experiment was repeated at least three times, and data are expressed as means.

For evoking the calcium response, 30 μM MeSADP, 100 μM UTP or 10 μM ADP was used as described by Sabala et al. [12]. For inhibition of $P2Y_{12}$, 10 μM AR-C69931MX was used, and 30 μM MRS 2179 was used for inhibition of $P2Y_1$ receptors.

Western blot analysis

For the experiments, cells were grown up to 85% confluency on 100-mm dishes in DMEM supplemented with 10% NCS. In order to cause serum deprivation, the medium was replaced by fresh serum-free medium for 96 h. After this time, cells were washed in PBS, detached with a

3-min CDS (cell dissociation solution) treatment, centrifuged at 3,000 rpm for 5 min and then resuspended in lysis buffer containing 1% Nonidet P-40, 120 mM NaCl, 50 mM Tris/HCl, pH 7.5, and freshly added proteinase inhibitors. Separation of proteins was performed on 12% polyacrylamide SDS/PAGE. Proteins were transferred to nitrocellulose membranes (Hybond C, Amersham Pharmacia Biotech), blocked for 1 h at room temperature with 5% milk in PBS-T (phosphate-buffered saline pH 7.6/0.005% Tween 20) and incubated overnight at 4°C with the antisera against β-actin (Sigma) and $P2Y_1$, $P2Y_{12}$, and $P2Y_2$ (all 1:2,500, Alomone and Sigma Adrich) diluted in 1% nonfat milk in PBS-T.

The primary antibody reaction was followed by 2 h incubation with relevant secondary (1:2,500) antibody conjugated to HRP. Immunocomplexes were detected using the ECL-enhanced chemiluminescence detection system and membrane exposure to X-ray film. The molecular weight of proteins was estimated with prestained protein markers (Fermentas protein ladder). Band intensities were determined by densitometry analysis in an Ingenious station using provided programs. Only statistically significant immunoblot band intensity data are reported.

Statistical analysis

Nonparametric Mann-Whitney U-test was used to discriminate differences between calcium responses. Differences with $P<0.01$ were considered highly significant and marked with two asterisks on the plots, $P<0.001$ were marked three asterisks, the lack of statistically significant difference was marked with a minus sign.

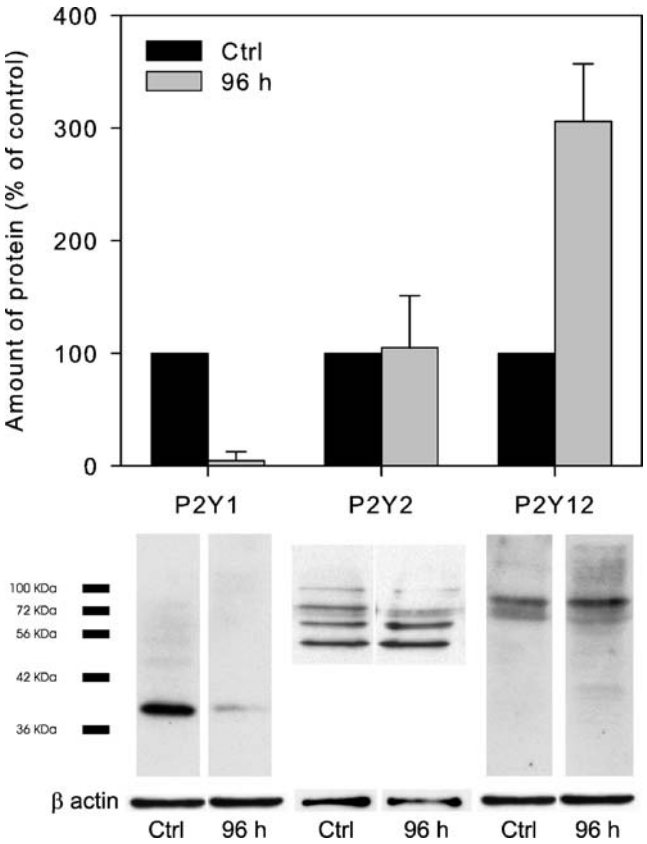

Fig. 1 The effect of long-term (96 h) serum deprivation on the relative expression levels of $P2Y_1$, $P2Y_2$ and $P2Y_{12}$ receptors. Total protein isolated from glioma C6 cells was subjected to SDS-PAGE on 12% acrylamide gel and transferred onto nitrocellulose membrane. Filters were probed with the indicated P2Y antisera. Protein bands were detected with secondary antisera coupled to HRP by ECL chemiluminescences. Band intensity was determined by densitometry analysis. The data represent the means ± SD from three independent experiments

Results

We have previously shown the presence of $P2Y_1$, $P2Y_2$ and $P2Y_{12}$ nucleotide receptors in glioma C6 cells using polymerase chain reaction on reverse-transcribed total mRNA [11, 12]. We have also shown that the mRNA expression level, as well as Ca^{2+} responses to extracellular nucleotides, may depend on the presence of serum in the cell-cultured medium. In cells that have been starved of serum for 48 h, the expression of the $P2Y_1$ receptor mRNA decreases and $P2Y_{12}$ predominates [9, 10]. This change reflects the lower intensity of Ca^{2+} response [9]. Furthermore, we have presented experimental data that the serum-free chemically defined medium used by other authors [18] causes similar changes, decreasing the expression of $P2Y_1$ receptor mRNA [10]. These data show that observed changes are present at the gene expression level.

In the present study, we explored the effects of prolonged serum deprivaton, up to 96 h, on the expression of the described receptors and measured changes in their protein level using Western blot analysis. Figure 1 shows that upon such treatment, the level of the $P2Y_1$ receptor protein was very low. Densitometry analysis of blots from three independent experiments revealed about a 20-fold decrease in this receptor protein level and a distinct, 3-fold increase in the $P2Y_{12}$ receptor.

When the level of P2Y protein receptors in the control C6 cells, cultivated in the medium supplemented with 10% new born calf serum, was taken as 100%, the level of $P2Y_1$ receptor protein in the 96-h-deprived serum cells was 6± 2%, and $P2Y_{12}$ was 306±60% (Fig. 1). Additionally, we have shown that serum deprivation had no effect either on $P2Y_2$ mRNA expression [9] or on total receptor protein level (Fig. 1). Multiple bands observed for P2Y receptors are probably the effect of protein glycosylation and were shown to disappear after total receptor deglycosylation [22]. We investigated such phenomena in glioma C6 cells and recently showed that preincubation of lysates of cells

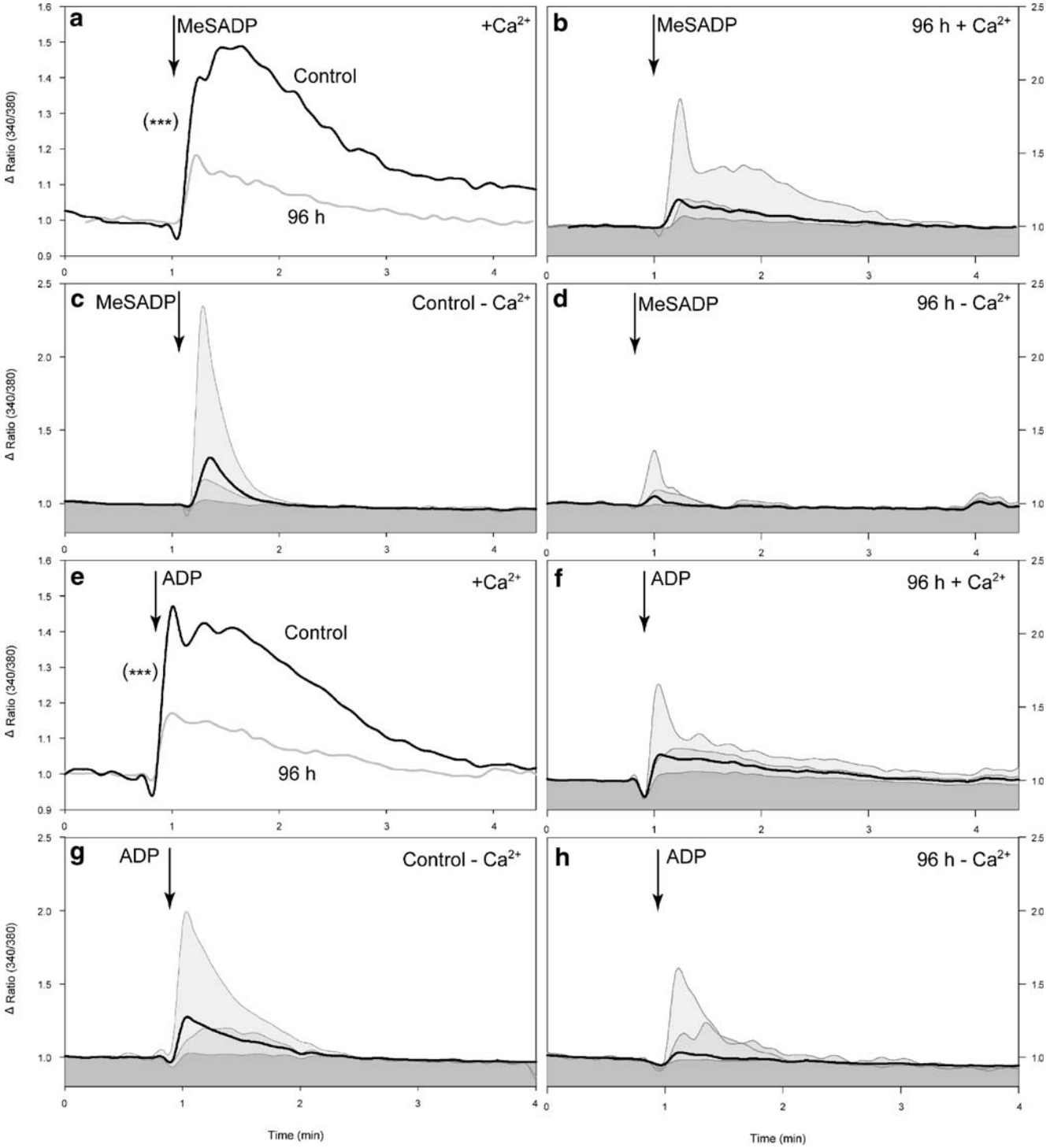

Fig. 2 a–h The effect of the long-term, 96-h serum deprivation on 2MeSADP-evoked calcium signals. The glioma C6 cells growing in the medium supplemented with 10% newborn calf serum (control cells) and those cultivated without serum for 96 h were loaded with Fura-2 and treated with 30 μM 2MeSADP or 10 μM ADP, as indicated by *arrows*. **a**, **b**, **e**, **f** Experiments performed in the standard buffer containing 2 mM $CaCl_2$. **c**, **d**, **g**, **h** Experiments conducted in the absence of extracellular Ca^{2+}. Each *trace* represents the mean ratio value of the responses of the indicated number of cells (*n*), recorded in five separate experiments. **a** *Black line* Control cells induced by 2MeSADP, $n=71$; *gray line* 96-h starved cells, $n=79$. **b** Ca^{2+} responses in 96-h serum-deprived cells induced by 2MeSADP, $n=79$, divided into groups according to the extent of response (*light gray*: cells with strong response, *medium gray*: cells with medium response, *dark gray*: cells with weak response). *Black line* Mean ratio value. **c** Control cells induced by 2MeSADP, $n=51$. **d** 96-h starved cells induced by 2MeSADP, $n=49$. **e** *Black line* Control cells induced by ADP, $n=51$; *gray line* 96-h starved cells, $n=134$. **f** Ca^{2+} responses in 96-h serum-deprived cells induced by ADP, $n=134$, divided into groups according to the extent of response (*light gray* cells with strong response, *medium gray* cells with medium response, *dark gray* cells with weak response). *Black line* Mean ratio value. **g** Control cells induced by ADP, $n=97$. **h** 96-h starved cells induced by ADP, $n=235$. ***$P<0.001$

with N-glycolidase F resulted in reduction of bands detected by P2Y antibodies [23].

Since under long-term serum starvation the $P2Y_{12}$ receptor expression strongly predominates, one could suppose that this receptor might be primarily involved in ADP- or 2MeSADP-evoked signal transduction. Slegers group [18] suggested the existence of an alternative, IP_3-independent, calcium influx pathway caused by $P2Y_{12}$ receptor activation [19, 20].

The fact that in long-term serum-deprived C6 glioma cells the $P2Y_{12}$ receptor is massively expressed and $P2Y_1$ strongly diminished provides a good model system to study functional properties of both receptors. Therefore, in our subsequent experiments, Ca^{2+} responses to nucleotides in the control cells were compared to those in the cells growing 96 h in the medium without serum.

Figure 2 shows the effect of 30 μM 2MeSADP (Fig. 2a–d) and 10 μM ADP (Fig. 2e–h) on Ca^{2+} response in the control, nonstarved cells, and in the 96-h serum-starved cells. Figure 2a, b, e, f shows cells studied in the extracellular medium containing 2 mM $CaCl_2$, while Fig. 2c, d, g, h shows calcium transients in cells tested in the medium containing 500 μM EGTA. Statistical analysis (mean value ± SD from at least three separate experiments for the indicated number of cells; data were standardized to obtain stationary calcium levels equal 1 arbitrary unit, AU)

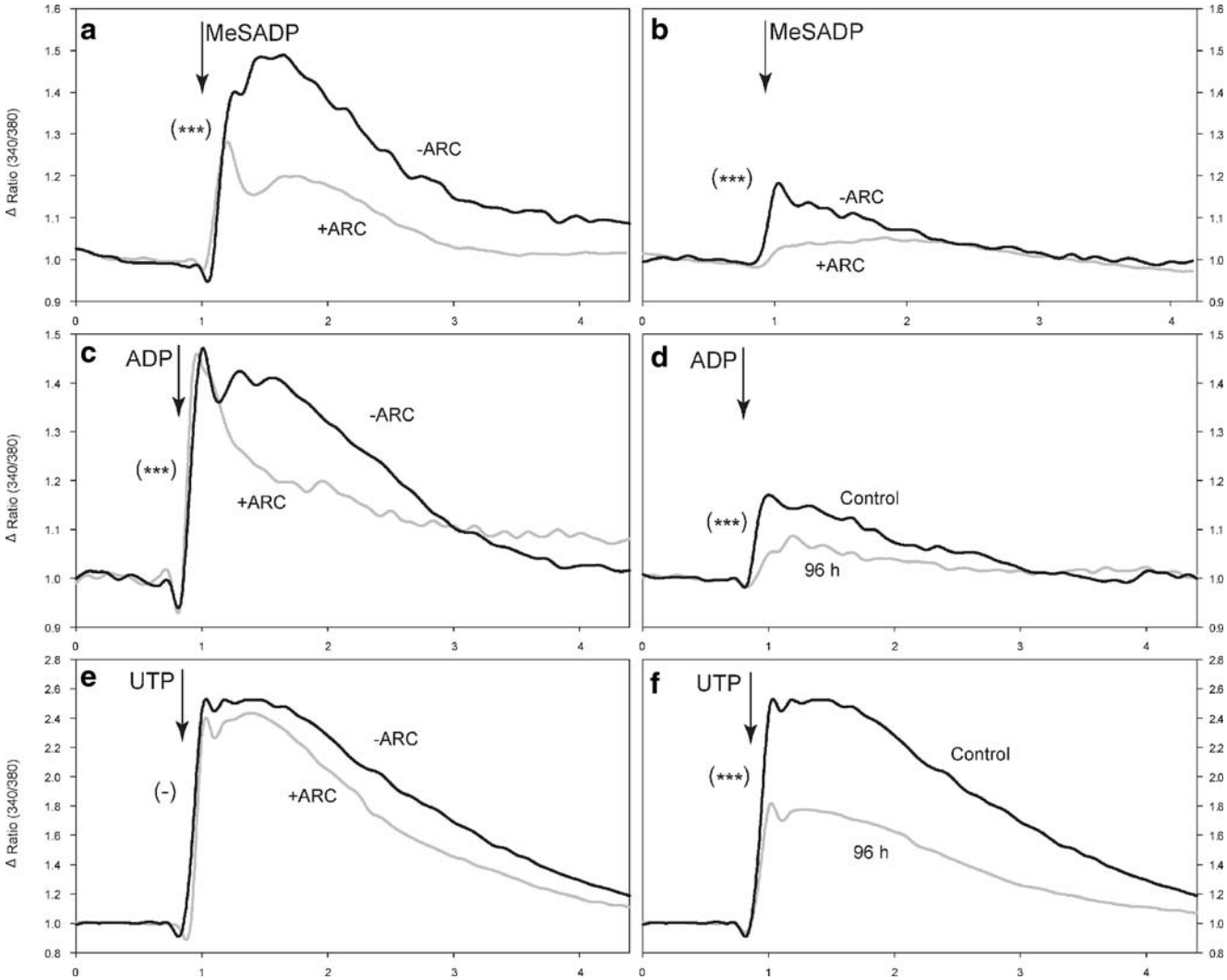

Fig. 3 The effect of AR-C69931MX, the $P2Y_{12}$ receptor antagonist, on Ca^{2+} signals evoked by 30 μM 2MeSADP (**a**, **b**), 10 μM ADP (**c**, **d**), and 100 μM UTP (**e**, **f**). *Black lines* represent mean ratio values of Ca^{2+} responses of cells untreated with $P2Y_{12}$ antagonist. **a**, **c**, **e** Nonstarved cells, respectively, n=71, n=51, n=73. **b**, **d** 96-h serum-starved cells, respectively, n=79, n=134. *Gray lines* in the same panels represent mean ratio value of Ca^{2+} responses of cells pre-treated for 3 min with 10 μM AR-C69931MX (**a**, n = 95; **b**, n=80; **c**, n=19 ; **d**, n=175; **e**, n=79) and then, while still in its presence, stimulated by agonist, as indicated by *arrows*. **f** 100 μM UTP-evoked Ca^{2+} signals in the control cells (n=73, *black line*); and 96-h serum-starved cells, (n=120, *gray line*). Each *trace* represents mean ratio value from five experiments. ***P<0.001. Statistically insignificant differences are indicated with a *minus sign*

revealed that in the control cells the initial Ca^{2+} peak evoked by 2MeSADP (Fig. 2a) and ADP (Fig. 2e) were 1.50±0.15 AU for 2MeSADP (n=71) and 1.47±0.37 AU for ADP (n=51), whereas the values in serum-starved cells were 1.19±0.21 AU for 2MeSADP (n=79) and 1.17± 0.25 AU for ADP (n=134). The difference was statistically significant with P<0.001.

The calcium signal strength thus fell to ~0.2 AU from ~0.5 AU indicating 60% inhibition of the signal. The initial calcium transient was associated with the depletion of intracellular stores (the first phase of the calcium response), followed by sustained elevation of Ca^{2+} (the second phase of the calcium response), which was a result of a subsequent influx of extracellular Ca^{2+} to the cells.

It is worth adding that Ca^{2+} responses are often analyzed only in cells that respond to agonists. We have previously shown that 70–95% of glioma C6 cells, growing in the medium supplemented with serum responded to extracellular nucleotides [12]. Similarly, of the control cells presented in Fig. 2a and e, 80% responded to agonist. In contrast, among serum-deprived cells, only 8.8% (7 out of 79) responded to 2MeSADP, and 16.4% responded to ADP (22 out of 134). Therefore, the traces shown in Fig. 2a and e represent mean values of all individual cell responses, including those responding and those not responding to the agonist.

Figure 2b and f shows Ca^{2+} responses in 96-h serum-deprived cells, divided into groups according to the extent of the response. It is shown that in the cells with the highest response, a rapid rise in intracellular Ca^{2+} level associated with the depletion of the ER stores is emphasized (the first phase), whereas the second phase is diminished. Figure 2c, d, g and h shows Ca^{2+} responses in the absence of extracellular Ca^{2+}. It is shown that addition of agonist resulted only in the initial transient rise in intracellular Ca^{2+} concentration, which declined to the basal level (the first phase of the calcium response) indicating that this phase of the cytosolic Ca^{2+} increase was indeed caused by mobilization of intracellular Ca^{2+} stores. The mean values of Ca^{2+} elevation in control cells were 1.30±0.17 AU (n=51, Fig. 2c) for 2MeSADP and 1.25±0.56 AU (n=97, Fig. 2g) for ADP; the values in serum-starved cells were 1.05±0.13 AU (n=49, Fig. 2d) for 2MeSADP and 1.03± 0.27 AU (n=235, Fig. 2h) for ADP. Among serum-deprived cells, 10% (5 out of 49) responded to 2MeSADP and 7% (17 out of 235) to ADP.

Thus, the very low expression of the $P2Y_1$ receptor in long-term serum-deprived cells strictly corresponds to the low intensity of the functional effects. When, in serum-starved cells, the $P2Y_1$ receptor protein level decreases to 6% of that in the control cells, this change is reflected by approximately 10% of the cells strongly responding to 2MeSADP or ADP. Since in serum-starved cells the expression of $P2Y_{12}$ receptor protein distinctly increases, low calcium responses reflect stimulation of $P2Y_1$ and not of $P2Y_{12}$ receptor. Nevertheless, the average calcium signal reduction is much weaker than can be expected from 94% reduction of $P2Y_1$ receptor protein level.

To check the possibility that $P2Y_{12}$ may still play a role in contributing to the agonist-induced Ca^{2+} response, the extent of inhibition of this response by the $P2Y_{12}$ selective antagonist AR-C69931MX (10 μM) was determined. Figure 3 shows that both in the control, nonstarved (Fig. 3a, c) and in the serum-deprived (Fig. 3b, d) cells, AR-C69931MX reduced the strength of the calcium response (measured as the signal integral) to 2MeSADP by 42.3% (Fig. 3a) and 69.8% (Fig. 3b), respectively, and to ADP by 31.9% (Fig. 3c) and 57.3% (Fig. 3d). In both cases the difference was statistically significant with P< 0.005. It is important to notice that, as shown in Fig. 3a and c, the observed difference in calcium signal integral caused by AR-C69931MX affected the second phase of calcium response. Since the plots presented in Fig. 3 show the overlay, we can not precisely state whether or not the fall of the initial peak in Fig. 3a is the result of the strong second-phase reduction.

In order to demonstrate that AR-C69931MX has no effect upon other P2Y receptors, Ca^{2+} responses were evoked by UTP (100 μM), an agonist of the $P2Y_2$ receptor. In these cases, there was no statistically significant change in the peak response either in the presence or absence of this antagonist (Fig. 3e). It is worth adding that MRS2179, the $P2Y_1$ receptor antagonist, led to a 96% reduction in calcium response induced by ADP [10, 11], or 75% reduction if induced by 2MeSADP (data not shown). Figure 3f also shows that the UTP-generated Ca^{2+} response is diminished in the 96-h serum-starved cells. In the control cells, the initial peak of Ca^{2+} elevation was 2.50±0.23 AU (n=73), whereas in the serum-deprived cells it was 1.58± 0.16 AU (n=120). The difference was statistically significant with P<0.001. Thus, 96-h, prolonged cell cultivation in the medium without serum blocked the effect of UTP by approximately 47.4%. On the other hand, serum-deprivation did not decrease the expression level of $P2Y_2$ receptor protein (Fig. 1).

Figure 4a shows two phases of calcium response in glioma C6 cells. The signal was evoked by UTP in the Ca^{2+}-free medium. Under such conditions, the rise in $[Ca^{2+}]_i$ was the effect of depletion of intracellular stores (ER). When the transient rise in calcium returned to the basal level, a standard, calcium-containing buffer was added. The addition of Ca^{2+} to the extracellular medium generated a high elevation in the cytosolic Ca^{2+} concentration. These data demonstrate that the Ca^{2+} entry is solely activated by the depletion of the ER Ca^{2+} store.

However, when we compared UTP-induced Ca^{2+} responses in the control (1.95±0.27 AU, n=95) and

serum-starved cells (0.52±0.18 AU, n=83), tested in Ca^{2+}-free medium, there was a visible difference in the response of both groups. The response to UTP in 96-h serum-deprived cells was much lower, and the difference was statistically significant with P<0.001. On the other hand, after exchanging that medium for one containing Ca^{2+}, the strong and rapid elevation of the cytosolic Ca^{2+} level was the same in both control (1.92±0.28 AU, n=95) and serum-deprived (1.83±0.24 AU, n=83) cells (Fig. 4a); the difference was statistically insignificant.

Please note that the data suggested that the different response to UTP in Ca^{2+}-free medium observed in the two groups of cells (see also Fig. 3d) not only depended on the expression of physiologically active receptors (which in this case was on the same level in both starved and nonstarved groups of cells) but also on the amount of Ca^{2+} present in the intracellular store. To check this assumption, 96-h serum-starved cells were placed in a buffer without Ca^{2+} for about 8 min, and then the buffer was changed for that containing 2 mM $CaCl_2$. This change resulted in a rapid increase in $[Ca^{2+}]_i$ caused by extracellular Ca^{2+} influx (1.28±0.14 AU, n=114) (Fig. 4b). Thus, prolonged, 96-h serum deprivation constitutes inhospitable conditions, manifested not only in the decrease in the $P2Y_1$ receptor expression but also in a partial, autonomous depletion of ER stores from Ca^{2+}. Nonstarved cells did not respond by increasing $[Ca^{2+}]_i$ when there was an exchange from calcium-free medium with that containing 2 mM $CaCl_2$ (Fig. 4b, n=36).

Therefore, in the long-term serum-starved glioma C6 cells tested in Ca^{2+}-free buffer, there was a very small, but statistically significant (P<0.001) increase in $[Ca^{2+}]_i$ (0.31±0.11 AU, n=111, Fig. 4c) induced by 2MeSADP. It was caused by both the low expression of the $P2Y_1$ receptor protein level and by a partial depletion of ER stores from Ca^{2+} (Figs. 2d and 4c). The change in the extracellular medium for the buffer containing Ca^{2+} caused Ca^{2+} influx to the cells (Fig. 4c). The influx after 96 h of serum starvation was even stronger (1.93±0.31 AU, n=107) than in control cells (1.43±0.24 AU, n=111); the difference was statistically significant with P<0.01. It is the depletion of ER stores that gives a signal to voltage-independent Ca^{2+} channels and Ca^{2+} entry across plasma membranes, according to the mechanism of the capacitative Ca^{2+} entry.

In subsequent experiments, the role of $P2Y_{12}$ on the capacitative Ca^{2+} entry was examined in the nonstarved cells. In such experiments the cells were incubated in the medium without Ca^{2+} (Fig. 5a). After addition of ADP, the first phase of calcium response was observed. Thereafter the medium was replaced by standard buffer containing 2 mM $CaCl_2$ (with no agonist).

To distinguish if the $P2Y_{12}$ receptor plays an active role in the calcium signal formation or if it only modulates the result of $P2Y_1$ receptor activity, we used AR-C69931MX in two separate experimental setups. In the first experiment, $P2Y_{12}$ receptor competitive antagonist was used before addition of agonist to inhibit both hypothetical $P2Y_{12}$ direct calcium signalling as well as regulation of $P2Y_1$ activity by $P2Y_{12}$ receptor. In the second experiment, the use of antagonist well after agonist addition but before medium

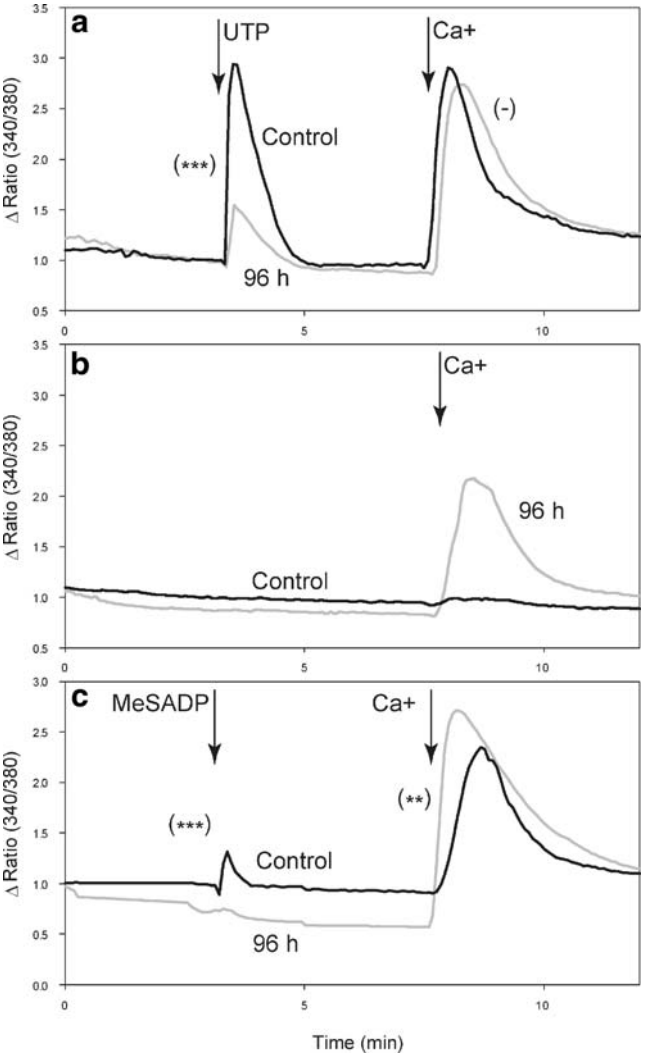

Fig. 4 a–c The effect of intracellular store depletion evoked by UTP, 2MeSADP, or by incubation in the calcium-free buffer on Ca^{2+} influx in glioma C6 cells. **a** 100 μM UTP was added to the control cells (*black line*, n=73), or to 96-h serum-starved cells (*gray line*, n=120), placed in a buffer without Ca^{2+} and with 500 μM EGTA. Next, 5 min after the addition of UTP, the buffer was changed for the buffer containing 2 mM $CaCl_2$ (Ca^{2+}). **b** The control cells (*black line*, n=30) and 96-h serum-starved cells (*gray line*, n=114) were placed in a buffer without Ca^{2+} and with 500 μM EGTA, and after 8 min incubation in the Ca^{2+}-free buffer, new buffer containing 2 mM $CaCl_2$ (Ca^{2+}) was added. **c** 30 μM 2MeSADP was added to the control cells (*black line*, n=78) or to 96-h serum-starved cells (*gray line*, n=92) placed in a buffer without Ca^{2+} and with 500 μM EGTA; 5 min after the addition of 2MeSADP, the buffer was changed for the buffer containing 2 mM $CaCl_2$ (Ca^{2+}). Each trace in **a**, **b**, and **c** represents the mean value for the indicated number of cells tested in three to five experiments. *** P<0.001, **P<0.01. Statistically insignificant differences are marked with a *minus sign*

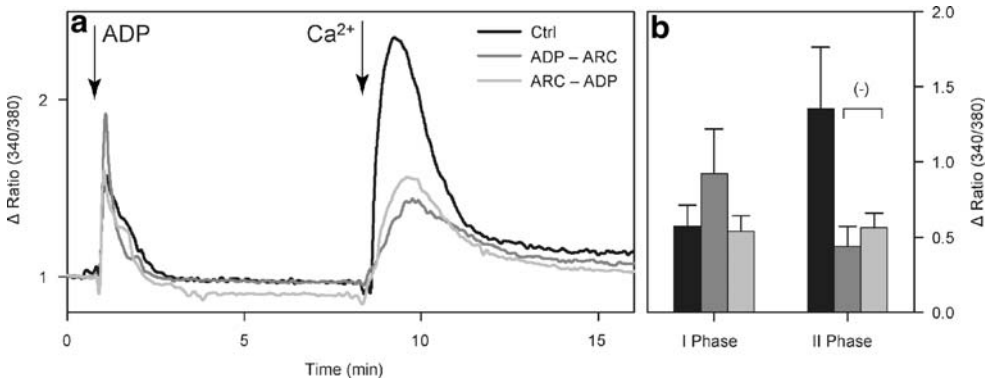

Fig. 5 a, b The effect of AR-C69931MX, the $P2Y_{12}$ receptor antagonist, on the first and second phase of calcium response in cells grown in the presence of serum. **a** Cells in Ca^{2+}-free buffer were stimulated by 10 μM ADP (*arrow*). Two cell groups were studied: the first experimental group was treated with 10 μM AR-C69931MX for 3 min before ADP addition and release of calcium from the ER (*light gray line*, ARC - ADP), while the second experimental group was treated for 3 min with 10 μM AR-C69931MX just after the end of calcium transient caused by ADP-induced release from the ER (*dark gray line*, ADP - ARC). The control cell group was induced with ADP, without use of AR-C69931MX (*black line*, Ctrl). Then, in all cell groups, the medium was exchanged with fresh medium containing 2 mM $CaCl_2$ (*arrow*). **b** Strength of two phases of calcium response. *Colour of bars* as described above. Difference between bars marked with a *minus sign* is statistically insignificant

replacement with that containing calcium should affect only regulatory functions of $P2Y_{12}$ receptor but not its ability to directly form the calcium signal.

As has been shown, AR-C69931MX has an inhibitory effect on the second phase of Ca^{2+} response, however this effect does not depend on the moment of the antagonist addition (cells treated with AR-C69931MX before ADP addition: 1.43±0.44 AU, n=12; cells treated with AR-C69931MX after ADP addition: 1.55±0.42 AU, n=18; the difference was not statistically significant) (Fig. 5b). Figure 5b also shows that AR-C69931MX has no statistically significant effect on the first phase of the calcium response. The same results were observed when 2MeSADP was used as an agonist (data not shown).

Discussion

It is now well-documented that in the G_q-dependent signalling initiated by ADP or 2MeSADP, the $P2Y_1$ receptor stimulation triggers PLCβ activation and $[Ca^{2+}]_i$ increase [9–17]. On the other hand, the same agonists, via the $P2Y_{12}$ receptor, activate the G_i pathway and inhibit adenylate cyclase in various animal cells [11, 12, 18, 24, 25]. The cross-talk between those two receptors is extremely complex [9, 26]. In human platelets, Sage et al. [27] and Fox et al. [28] suggested that $P2Y_{12}$ may enhance $P2Y_1$-induced cytosolic Ca^{2+} rise, whereas Daniel et al. [29] presented evidence that this receptor is not involved in such response. Hardy et al. [30] have explained this conflicting evidence as the different conditions used during platelets preparation. Similarly in glioma C6 cells, there is conflicting evidence regarding the role of $P2Y_1$ in ADP-mediated calcium response that can also be explained by the differences in the culture conditions [10]. Presence or absence of serum in the culture medium provides conclusions on functional activity [9–11] or inactivity [18] of this receptor.

Hardy et al. [30], as well as Sage et al. [27], suggested the modulatory role of $P2Y_{12}$, positively regulating $P2Y_1$-induced Ca^{2+} response. It has been suggested that this potentiation is mediated by $P2Y_{12}$-induced inhibition of adenylate cyclase and activation of phosphatidylinositol 3-kinase (PI3-K), whereas the effect of $P2Y_1$ on PI3-K is inhibitory [30]. Our previous study concerning cross-talk between nucleotide receptor-induced signalling pathways in glioma C6 cells also revealed $P2Y_1$ inhibitory and $P2Y_{12}$ stimulatory effects on PI3-K signalling [9, 10]. Thus, since stimulation of the $P2Y_{12}$ receptor in glioma C6 inhibits adenylate cyclase [11, 12] and stimulates PI3-K [9, 10], its modulatory effect on the $P2Y_1$-induced Ca^{2+} responses in this cell line may occur via a similar mechanism to the one suggested in platelets [30]. It has been proposed that in this process the cAMP-dependent pathway has a stimulatory effect on PM calcium pumps, thereby limiting the strength of the calcium response. The $P2Y_{12}$ receptor reduced this effect by inhibition of adenylate cyclase activity. Hardy et al. [30] suggested that PI3-K, activated by the $P2Y_{12}$ receptor stimulation, may also activate PLCγ, leading to the rise in PIP_3 and enhancement of the calcium signal. It has been reported that receptors coupled to PI3-K may activate PLCγ indirectly in the absence of PLCγ-tyrosine phosphorylation [31, 32].

Our study confirms the conclusions of Hardy et al. [30] concerning the positive regulatory role of $P2Y_{12}$ potentiating the $P2Y_1$-induced rise in intracellular Ca^{2+} level. We have shown here that, in glioma C6, the calcium response to ADP or its analogue 2MeSADP is directly dependent

upon activation of the $P2Y_1$ receptor. The modulatory effect of $P2Y_{12}$ receptor on intracellular free calcium level seems to be a result of the PM calcium pump inhibition [26]. The pump activity is enhanced by cAMP. The constant activation of $P2Y_{12}$ receptor lowers the level of cAMP and inhibits the PMCA pump. Thus, inhibition of this receptor activity may enhance the pump efficiency and lower the calcium signal as shown in the present study when we observed decreased calcium influx under the influence of the $P2Y_{12}$ competitive antagonist AR-C69931MX (Fig. 5). A similar phenomenon of calcium-removal inhibition was described in platelets, where the adenylate cyclase inhibitor SQ22536 reduced the strength of calcium signal by approximately 50% [26].

Thus, the suggestion that Ca^{2+} influx in this cell line may involve a channel, activated directly or indirectly by protein $G\beta\gamma$ subunits after the $P2Y_{12}$ receptor stimulation (see figures in [19, 20]), seems to be doubtful. If such a channel were to exist it should be opened by an agonist when $P2Y_{12}$ antagonist is added after addition of the agonist and remain closed if $P2Y_{12}$ antagonist is added before this agonist (Fig. 5). Since we did not observe such a difference in the calcium signal strength we may exclude the existence of a $P2Y_{12}$-dependent calcium channel in glioma C6 cells. Even if there is some evidence that the stimulation of P2Y receptors may affect N-type Ca^{2+}currents [33], glioma C6 cells belong to the type of nonexcitable cells and do not possess voltage-dependent Ca^{2+} channels [3].

In conclusion, the present study shows that Ca^{2+} influx in these cells occurs exclusively via the mechanism of capacitative calcium entry. Thus any activation of calcium-dependent signalling pathways resulting from the presence of extracellular ADP must be the result of $P2Y_1$-$P2Y_{12}$ cross-talk, not solely $P2Y_{12}$ receptor activity. Nevertheless, the $P2Y_{12}$ modulatory effect on glioma C6 calcium signalling seems to be very important.

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References

1. Clapham DE (1995) Calcium signaling. Cell 80:259–268
2. Fasolato C, Innocenti B, Pozzan T (1994) Receptor-activated Ca2+ influx: how many mechanisms for how many channels? Trends Pharmacol Sci 15:77–83
3. Baranska J, Chaban V, Czarny M, Sabala P (1995) Changes in Ca2+ concentration in phorbol ester and thapsigargin treated glioma C6 cells. The role of protein kinase C in regulation of Ca2+ entry. Cell Calcium 17:207–215
4. Baranska J, Przybylek K, Sabala P (1999) Capacitative calcium entry. Glioma C6 as a model of nonexcitable cells. Pol J Pharmacol 51:153–162
5. Berridge MJ (1993) Inositol trisphosphate and calcium signalling. Nature 361:315–325
6. Berridge MJ (1995) Capacitative calcium entry. Biochem J 312 (Pt 1):1–11
7. Putney JW Jr (1986) A model for receptor-regulated calcium entry. Cell Calcium 7:1–12
8. Putney JW Jr, Bird GS (1993) The inositol phosphate-calcium signaling system in nonexcitable cells. Endocr Rev 14:610–631
9. Baranska J, Czajkowski R, Sabala P (2004) Cross-talks between nucleotide receptor-induced signaling pathways in serum-deprived and non-starved glioma C6 cells. Adv Enzyme Regul 44:219–232
10. Czajkowski R, Banachewicz W, Ilnytska O, Drobot LB, Baranska J (2004) Differential effects of P2Y1 and P2Y12 nucleotide receptors on ERK1/ERK2 and phosphatidylinositol 3-kinase signalling and cell proliferation in serum-deprived and nonstarved glioma C6 cells. Br J Pharmacol 141:497–507
11. Czajkowski R, Lei L, Sabala P, Baranska J (2002) ADP-evoked phospholipase C stimulation and adenylyl cyclase inhibition in glioma C6 cells occur through two distinct nucleotide receptors, P2Y(1) and P2Y(12). FEBS Lett 513:179–183
12. Sabala P, Czajkowski R, Przybylek K, Kalita K, Kaczmarek L, Baranska J (2001) Two subtypes of G protein-coupled nucleotide receptors, P2Y(1) and P2Y(2) are involved in calcium signalling in glioma C6 cells. Br J Pharmacol 132:393–402
13. Sak K, Illes P (2005) Neuronal and glial cell lines as model systems for studying P2Y receptor pharmacology. Neurochem Int 47:401–412
14. Illes P, Ribeiro JA (2004) Neuronal P2 receptors of the central nervous system. Curr Top Med Chem 4:831–838
15. Burnstock G (1978) A basis for distinguishing two types of putinergic receptor. In: Straub R, Bolins L (eds) Cell membrane receptors for drugs and hormones. Raven Press, New York, pp 107–118
16. Boeynaems JM, Communi D, Savi P, Herbert JM (2000) P2Y receptors: in the middle of the road. Trends Pharmacol Sci 21:1–3
17. Abbracchio MP, Burnstock G (1994) Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther 64:445–475
18. Grobben B, Claes P, Van Kolen K, Roymans D, Fransen P, Sys SU, Slegers H (2001) Agonists of the P2Y(AC)-receptor activate MAP kinase by a ras-independent pathway in rat C6 glioma. J Neurochem 78:1325–1338
19. Van Kolen K, Gilany K, Moens L, Esmans EL, Slegers H (2006) P2Y(12) receptor signalling towards PKB proceeds through IGF-I receptor cross-talk and requires activation of Src, Pyk2 and Rap1. Cell Signal 18:1169–1181
20. Van Kolen K, Slegers H (2006) Integration of P2Y receptor activated signal transduction pathways in G protein-dependent signalling networks. Purinergic Signalling 2:451–469
21. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450
22. Yoshioka K, Saitoh O, Nakata H (2002) Agonist-promoted heteromeric oligomerization between adenosine A(1) and P2Y(1) receptors in living cells. FEBS Lett 523:147–151
23. Krzeminski P, Misiewicz I, Pomorski P, Kasprzycka-Guttman T, Baranska J (2006) Mitochondrial localization of P2Y1, P2Y2 and P2Y12 receptors in rat astrocytes and glioma C6 cells. Brain Res Bull doi:10.1016/j.brainresbull.2006.11.013 (in press)
24. Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, Conley PB (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 409:202–207
25. Jin J, Tomlinson W, Kirk IP, Kim YB, Humphries RG, Kunapuli SP (2001) The C6-2B glioma cell P2Y(AC) receptor is pharmacologically and molecularly identical to the platelet P2Y(12) receptor. Br J Pharmacol 133:521–528

26. Czajkowski R, Baranska J (2002) Cross-talk between the ATP and ADP nucleotide receptor signalling pathways in glioma C6 cells. Acta Biochim Pol 49:877–889
27. Sage SO, Yamoah EH, Heemskerk JW (2000) The roles of P(2X1) and P(2T AC)receptors in ADP-evoked calcium signalling in human platelets. Cell Calcium 28:119–126
28. Fox SC, Behan MW, Heptinstall S (2004) Inhibition of ADP-induced intracellular Ca2+ responses and platelet aggregation by the P2Y12 receptor antagonists AR-C69931MX and clopidogrel is enhanced by prostaglandin E1. Cell Calcium 35:39–46
29. Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP (1998) Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. J Biol Chem 273:2024–2029
30. Hardy AR, Jones ML, Mundell SJ, Poole AW (2004) Reciprocal cross-talk between P2Y1 and P2Y12 receptors at the level of calcium signaling in human platelets. Blood 104:1745–1752
31. Bae YS, Cantley LG, Chen CS, Kim SR, Kwon KS, Rhee SG (1998) Activation of phospholipase C-gamma by phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 273:4465–4469
32. Falasca M, Logan SK, Lehto VP, Baccante G, Lemmon MA, Schlessinger J (1998) Activation of phospholipase C gamma by PI 3-kinase-induced PH domain-mediated membrane targeting. Embo J 17:414–422
33. Claes P, Slegers H (2004) P2Y receptor activation affects the proliferation and differentiation of glial and neuronal cells: a focus on rat C6 glioma cells. Curr Neuropharmacol 2:207–220