Chapter 3
Cross-Talk in Nucleotide Signaling in Glioma C6 Cells

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Abstract The chapter is focused on the mechanism of action of metabotropic P2Y nucleotide receptors: $P2Y_1$, $P2Y_2$, $P2Y_{12}$, $P2Y_{14}$ and the ionotropic $P2X_7$ receptor in glioma C6 cells. $P2Y_1$ and $P2Y_{12}$ both respond to ADP, but while $P2Y_1$ links to PLC and elevates cytosolic Ca^{2+} concentration, $P2Y_{12}$ negatively couples to adenylate cyclase, maintaining cAMP at low level. In glioma C6, these two P2Y receptors modulate activities of ERK1/2 and PI3K/Akt signaling and the effects depend on physiological conditions of the cells. During prolonged serum deprivation, cell growth is arrested, the expression of the $P2Y_1$ receptor strongly decreases and $P2Y_{12}$ becomes a major player responsible for ADP-evoked signal transduction. The $P2Y_{12}$ receptor activates ERK1/2 kinase phosphorylation (a known cell proliferation regulator) and stimulates Akt activity, contributing to glioma invasiveness. In contrast, $P2Y_1$ has an inhibitory effect on Akt pathway signaling. Furthermore, the $P2X_7$ receptor, often responsible for apoptotic fate, is not involved in Ca^{2+}elevation in C6 cells. The shift in nucleotide receptor expression from $P2Y_1$ to $P2Y_{12}$ during serum withdrawal, the cross talk between both receptors and the lack of $P2X_7$ activity shows the precise self-regulating mechanism, enhancing survival and preserving the neoplastic features of C6 cells.

Keywords $P2Y_1$, $P2Y_2$, $P2Y_{12}$, $P2Y_{14}$, $P2X_7$ nucleotide receptors • Serum withdrawal • $P2Y_1$/$P2Y_{12}$ cross-talk • cAMP • ERK1/2, PI3K/Akt activity • Glioma C6 cells

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Abbreviations

Akt	Protein kinase B/Akt kinase
Ap_4A	p^1,p^4-Di(adenosine-5′)tetraphosphate
BzATP	2′,3′-*O*-(4-Benzoylbenzoyl)-ATP
$[Ca^{2+}]_i$	Intracellular calcium concentration
DAG	Diacylglycerol
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinases 1/2
Gap1	GTP-ase activating protein 1
GBM	*Glioblastoma multiforme*
GEF	Guanine nucleotide exchange factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
IP_3	Inositol-1,4,5-trisphosphate
LPS	Lipopolysacharide
MEK	MAP kinase-ERK kinase
2MeSADP	2-Methylthio ADP
OxATP	Periodate-oxidized ATP
PDK1	Phosphoinositide-dependent protein kinase 1
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PPADS	Pyridoxal-phosphate-6-azophenyl-2′,4′disulfonic acid
PTEN	Phosphatase and tensin homolog
PTX	Pertussis toxin
RTK	Receptor tyrosine kinase

3.1 Introduction

Gliomas are the most common brain tissue primary tumors that originate from glial cells. One of the most malignant form of gliomas, resistant to therapeutic intervention, is human *glioblastoma multiforme* (GBM) characterized by a marked increase in cell proliferation and invasiveness (Barth 1998; Collins 1998; Grobben et al. 2002; Salcman 1995). There are many models used in studies of glioma cell biology. One of them, widely used in GBM research, is rat glioma C6 cells. This tumoral cell line was originally produced in Wistar-Furth rats exposed to N,N′-nitroso-methylurea (Benda et al. 1968). Glioma C6 cells have oligodendrocytic as well as astrocytic progenitor properties and are also often used as a biochemical model for studies related to astrocytes (Brismar 1995). Moreover, the cells injected into rodent brain become morphologically similar to GBM (Auer et al. 1981). They are used in both *in vivo* and *in vitro* studies of this kind of tumor (see Chap. 5) since brain cancer treatment are still needed.

Recent evidence suggests that glioma development is determined by ATP signaling (White and Burnstock 2006). However, not only ATP but also UTP as well as their metabolites: ADP, UDP and adenosine have an influence on cell metabolism by stimulation of specific receptors present on the plasma membrane (see Chap. 1). This stimulation regulates proliferation, invasiveness and cell death. Thus, nucleotide receptors might be useful therapeutic targets (Burnstock 2002).

As described in Chap. 1, the large family of nucleotide receptors is divided into two major classes: P1, responding to adenosine, and P2, responding to ATP, UTP, ADP, UDP and UDP-sugar derivatives. To the P2 class belong: the intrinsic ligand-gated ion channels – P2X receptors and the metabotropic G-protein coupled P2Y receptors (Abbracchio and Burnstock 1994). To date, eight mammalian P2Y receptors have been cloned and pharmacologically characterized (see Fig. 1.1). Among them, $P2Y_2$ is activated by ATP and UTP, whereas $P2Y_1$ and $P2Y_{12}$ both respond to ADP. $P2Y_1$ and $P2Y_2$ are 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 (Ralevic and Burnstock 1998). However, one receptor can activate more than one signaling pathway. Such an example is a novel $P2Y_{14}$ receptor that specifically responds to UDP-glucose and related sugar-nucleotides (Abbracchio et al. 2003). Its stimulation leads both to calcium mobilization and to inhibition of adenylate cyclase. $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ are expressed in glioma C6 cells (Barańska et al. 2004; Braganhol et al. 2009; Czajkowski and Barańska 2002; Krzemiński et al. 2008; Van Kolen and Slegers 2006b). Contrary to P2Y receptors, the expression and activity of P2X receptors in glioma C6 cells have not been intensively examined. However, it was shown that the $P2X_7$ receptor, which responds to ATP is not active in this cell line (Supłat-Wypych et al. 2010).

This chapter is focused on the cross-talk between the metabotropic $P2Y_1$ and $P2Y_{12}$ nucleotide receptors. Their stimulation modulates activities of extracellular signal-regulated kinases (ERK1/2) and phosphatidylinositol 3-kinase/Akt kinase (PI3K/Akt) (Barańska et al. 2004). $P2Y_{12}$ not only reduces the level of cAMP in the cell but is also responsible for activating ERK1/2 kinase phosphorylation (a known cell proliferation regulator) and stimulating Akt activity (Czajkowski et al. 2004; Krzemiński et al. 2007). Thus, $P2Y_{12}$ acts in favor of cell growth and proliferation. In contrast, $P2Y_1$ has an inhibitory effect on the Akt signaling pathway. The above effects depend on the physiological conditions. During prolonged serum deprivation, $P2Y_1$ receptor expression is strongly decreased while that of $P2Y_{12}$ is distinctly increased in C6 cells. The intense pathological proliferation and invasiveness of glioma cells seems to depend on the constitutive low level of cAMP (Stork and Schmitt 2002) and up-regulation of the PI3K/Akt pathway (Kubiatowski et al. 2001). Thus, the shift in expression from $P2Y_1$ to $P2Y_{12}$ during serum starvation suggests the existence of a self-sufficient mechanism for survival of C6 cells under unfavorable conditions. Furthermore, it is well known that prolonged activation of the $P2X_7$ receptor is a common signal for apoptosis and cell death due to massive Ca^{2+} entry in many cells (Ferrari et al. 1997). The lack of such $P2X_7$ receptor activity in glioma C6 cells supports the hypothesis that gliomas are strongly oriented to pro-survival mechanism and intensive proliferation.

3.2 Properties of P2 Receptors

ATP release from neuronal cells and synaptic terminals occurs *via* exocytosis. However, recent findings indicate that both adenine and uridine nucleotides can be released from non-neuronal cells *via* regulated processes such as: exocytosis, secretion of granules, efflux, changes in osmolarity or mechanical perturbation. Moreover, in primary astrocytes, astrocytoma and glioma cells ATP release is accompanied by enhanced accumulation of extracellular UTP and this phenomenon is a result of the UDP-glucose release (for review see Lazarowski 2006).

ATP released to the extracellular space can exert its effects *via* activation of the plasma membrane P2 nucleotide receptors, both P2X and P2Y. Afterwards, ATP can also be degraded to ADP, AMP and adenosine by ecto-enzymes (ectonucleotidases) present on the cell surface (Zimmermann 2000; see also Chap. 5). To date, four subtypes of P1 adenosine receptors have been cloned: A_1, A_{2A}, A_{2B} and A_3. They all belong to the G protein-coupled metabotropic receptor superfamily and their effects are presented and discussed in Chap. 2 of this book. Here, we present the description of P2 receptors, whose activation plays a crucial role in glioma biology.

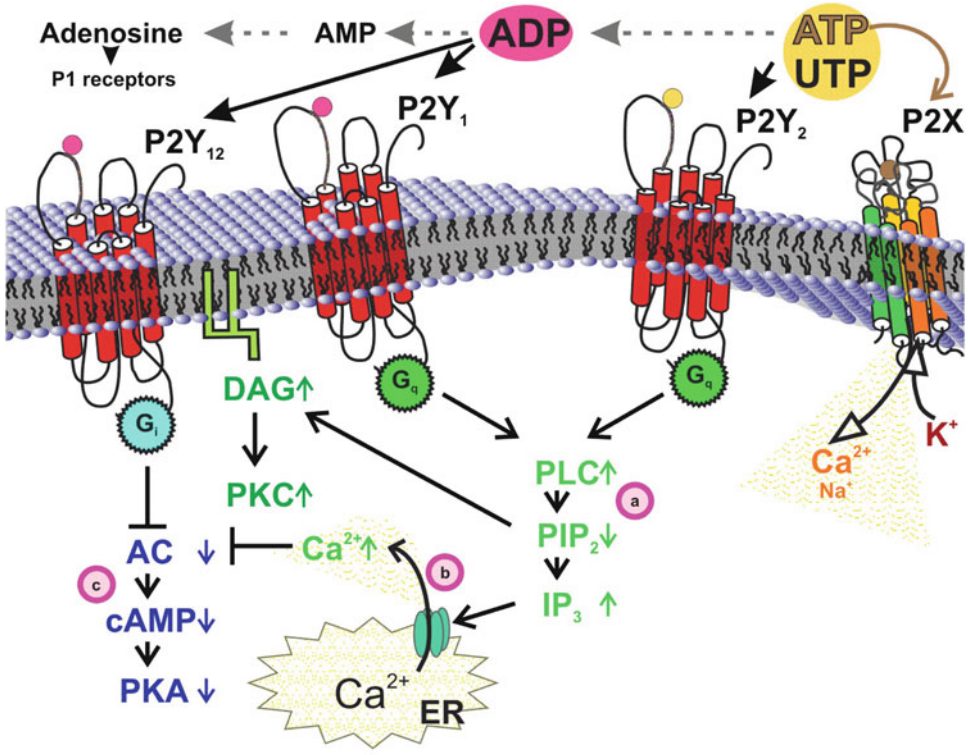
3.2.1 P2X Receptors

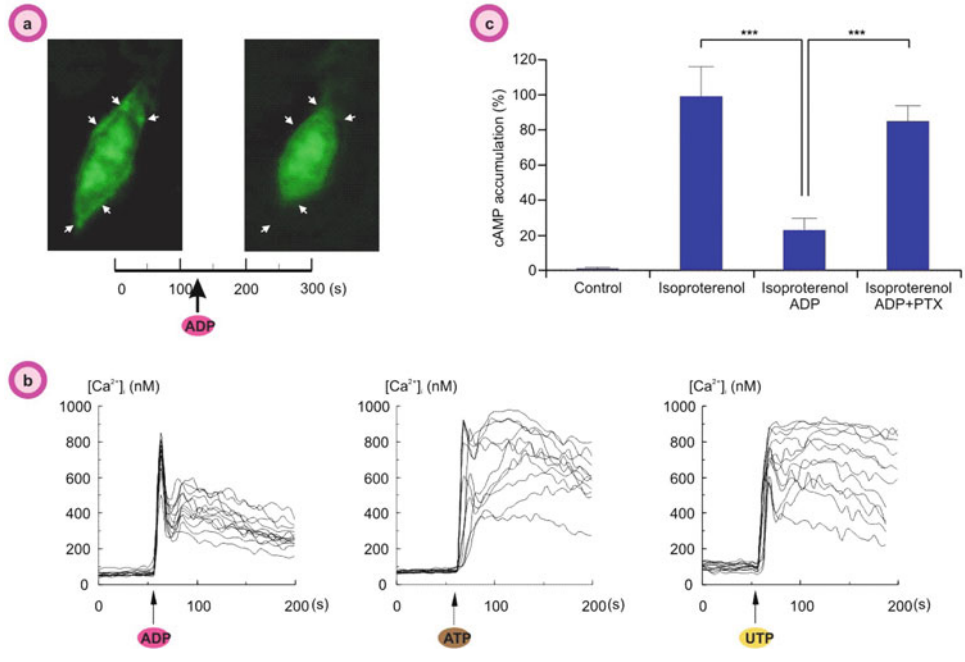
To date, seven mammalian P2X receptor subtypes ($P2X_1$ – $P2X_7$) have been cloned (for review see Burnstock 2007; Ralevic and Burnstock 1998). Ionotropic P2X receptors are ATP-gated, nonselective cation channels formed by three subunits (for review see North and Surprenant 2000; North 2002). Each subunit consists of two transmembrane domains with short intracellular NH_2 and COOH termini. These two segments are associated by a large extracellular N-glycosylated hydrophilic loop. One type or more than one type of subunits can form homotrimeric or heterotrimeric receptors, respectively. Activation of P2X receptors by ATP, but not UTP, produces rapid Ca^{2+} and Na^+ influx coupled with K^+ efflux (Fig. 3.1) and results in depolarization of cells. This, in turn, leads to opening of additional plasma membrane voltage-dependent calcium channels and Ca^{2+} flows in from the extracellular space. Thus, upon P2X receptor activation, opening of cation channels results in elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and in reduction of the membrane potential. Such effects are usually detected by properties of the unitary currents of ion channels in outside-out patches and by measuring increase of $[Ca^{2+}]_i$. The time course of calcium response is one of the main criteria distinguishing ionotropic (P2X) form metabotropic (P2Y) receptors. P2X are characterized by rapid, within milliseconds, $[Ca^{2+}]_i$ elevation while P2Y receptors response is much slower and occurs in minutes (North and Surprenant 2000; Ralevic and Burnstock 1998).

The P2X receptor family shows many pharmacological differences, however ATP is the main agonist of all P2X subunits and neither UTP nor UDP lead to their activation. Another ligand of several P2X receptor subunits is an analog of ATP, α,β-methylene ATP, although it is able to activate receptors in a different range (for more details see Burnstock 2007). Among members of the P2X receptor family the homomeric $P2X_7$ is atypical. It needs ATP concentration an order of magnitude higher (milimolar) than other P2X family members and is 10–30 times more sensitive to the ATP analog, 2′,3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP). Furthermore, activation of $P2X_7$ by milimolar concentrations of ATP for prolonged time generates the conversion of this cation channel to a large nonselective transmembrane pore. In consequence, massive Ca^{2+} influx is observed and small molecules (up to 900 Da in size) enter the cell. Due to such properties, $P2X_7$ receptors may be responsible for an apoptotic fate of the cell (Anderson and Nedergaard 2006; North 2002; White and Burnstock 2006).

P2X receptors are widely expressed in the body. They are present not only in excitable cells, such as neurons and skeletal muscle cells, but also in non-excitable ones, for example: smooth muscle, epithelial, endothelial or glial cells. Due to rapid response, they are largely viewed as mediators of fast, short-term neuronal signaling and cell-to-cell communication. P2X receptors also play an

Fig. 3.1 Schematic diagram of nucleotide signaling pathways activated in glioma C6 cells. *Upper part*: Effects of extracellular nucleotides on metabotropic (P2Y) and ionotropic (P2X) receptors. ATP stimulates P2X receptors and together with UTP acts on the $P2Y_2$ receptor. Ectoenzymes hydrolyze ATP to ADP and to adenosine, which acts on P1 receptors. ADP stimulates both $P2Y_1$ and $P2Y_{12}$ receptors. $P2Y_1$ and $P2Y_2$ activate PLC *via* protein G_q. (**a**) PLC converts PIP_2 to IP_3 and DAG, which activates PKC. (**b**) IP_3 binds to its receptors in the endoplasmic reticulum (ER) and causes release of Ca^{2+} to cytosol. Calcium inhibits adenylate cyclase (AC) activity. (**c**) The $P2Y_{12}$ receptor coupled to G_i protein inhibits AC activity, decreases intracellular level of cAMP and inhibits PKA activity. *Lower part*: Visualization of signaling cascades in glioma C6 cells described above. (**a**) PIP_2 breakdown activated by ADP *via* $P2Y_1$ receptor activation. PIP_2 is bound to GFP and fluorescence microscope pictures show the same cell *before* and *after* 5 min of ADP stimulation. *Arrows* mark points of PIP_2 breakdown. (**b**) Effect of ADP, ATP and UTP on $[Ca^{2+}]_i$ measured in Fura-2 loaded cells. Experiments performed in the presence of extracellular Ca^{2+}. Each trace represents the response of an individual cell. Notice a rapid rise in $[Ca^{2+}]_i$ (the first phase of the signal) upon addition of agonists (*arrows*), followed by a sustained elevation of $[Ca^{2+}]_i$ (the second phase of the signal). (**c**) ADP-induced inhibition of the isoproterenol-elicited cAMP response. Notice a very low cAMP level in control cells in the absence of isoproterenol. The inhibitory effect of ADP was reversed by PTX, a specific inhibitor of G_i protein, responsible for the inhibition of AC. For details, see text (Adapted from Czajkowski et al. (2002) (**a**) with permission from Elsevier and from Sabała et al. 2001 (**b** and **c**) with permission from John Wiley and Sons)


Adenosine
AMP
ADP
ATP
UTP
P1 receptors
P2Y12
P2Y1
P2Y2
P2X
Gi
Gq
Gq
DAG↑
PKC↑
PLC↑
a
PIP2↓
IP3 ↑
AC ↓
c
cAMP↓
PKA ↓
Ca2+↑
b
Ca2+
ER
Ca2+
Na+
K+


a
0
100
200
300 (s)
ADP
c
cAMP accumulation (%)
120
100
80
60
40
20
0
***
***
Control
Isoproterenol
Isoproterenol ADP
Isoproterenol ADP+PTX
b
[Ca2+] (nM)
1000
800
600
400
200
0
0
100
200(s)
ADP
[Ca2+] (nM)
ATP
[Ca2+] (nM)
UTP

important role in muscle development and fiber contraction. Ryten et al. demonstrated that in satellite cells activation of $P2X_5$ receptors inhibited proliferation and stimulated expression of muscle cell differentiation markers (Ryten et al. 2002). Similarly, results from our laboratory showed that in the C2C12 cell line, $P2X_5$ is involved in regulation of the shift between myoblast proliferation and differentiation (Banachewicz et al. 2005).

Fumagalli et al. presented evidence that primary rat cortical astrocytes express all cloned P2X receptors except for the $P2X_6$ subtype (Fumagalli et al. 2003). In contrast, glioma C6 cells growing *in vitro* express mRNA for the $P2X_2$, $P2X_4$ and $P2X_5$ subtypes. However, after implantation of the cells to rat brain and then their further growth as primary culture (*ex vivo* C6 model) only $P2X_4$ mRNA can be detected (Braganhol et al. 2009). Expression of $P2X_4$ was also reported in C6 glioma cells implanted into rat brain, where the presence of tumor cells was associated with microglia activation (Guo et al. 2004). $P2X_7$ receptors expressed in neuronal populations, due to their ability to massively increase the intracellular Ca^{2+} level, play a role in excitotoxicity. In various CNS injury models, e.g. *in vivo* spinal cord trauma, activation of $P2X_7$ receptors by a high level of released ATP, leads to death of neurons (Anderson and Nedergaard 2006). On the other hand, in glial cells with an immunocompetent role in CNS (microglia), $P2X_7$ receptors participate in controlling proliferation by supporting cell cycle progression (Bianco et al. 2006). The expression and functional activity of the $P2X_7$ receptors in glioma C6 cells will be discussed in further sections of this Chapter.

3.2.2 P2Y Receptors

Up to now, the mammalian P2Y receptor family comprises eight subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ (Abbracchio et al. 2003; Boarder and Webb 2001; Communi et al. 2001; Harden et al. 1998; Hollopeter et al. 2001; King et al. 2000; Zhang et al. 2002). The missing numbers represent receptors cloned from non-mammalian vertebrates and those not fully characterized, called "orphan" receptors.

Similarly to other metabotropic receptor proteins, P2Y contain seven transmembrane domains linked to each other by extra- and intra-cellular hydrophilic loops, the extracellular NH_2 terminus and intracellular COOH terminus (Burnstock 1997, 2007; Ralevic and Burnstock 1998). The schematic structure of the receptors is presented in Fig. 3.1. P2Y receptors act *via* binding to a single α,β,γ – heterotrimeric G protein. It is suggested that positively charged amino acid residues present in transmembrane regions 3, 6 and 7 form a ligand-docking pocket and are involved in electrostatic interaction with phosphate groups of exogenous ATP. This binding is affected by functional diversity of the intracellular loops and the COOH terminus (Ralevic and Burnstock 1998).

From a phylogenetic and structural point of view, the P2Y receptor family can be divided into two subgroups with relatively high structural divergence. The first one contains $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors, while to the other one belong $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors (Abbracchio et al. 2003). Each subgroup is characterized by specific nucleotide-binding motifs in transmembrane domains 6 and 7, considered as important for ligand binding and crucial for agonist activity (Abbracchio et al. 2003). All P2Y receptors classified to the first subgroup are coupled to $G_{q/11}$ protein. Their stimulation, *via* G_q protein, activates phospholipase C (PLC), which leads to hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) and to formation of diacylglicerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) (Boarder and Hourani 1998) (Fig. 3.1). DAG activates protein kinase C (PKC), while IP_3 binds to its specific receptors on the endoplasmic reticulum (ER) membrane, evoking Ca^{2+} release from the ER stores (Berridge 1995; Putney and Bird 1993). Taken together, stimulation of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors leads to mobilization of intracellular Ca^{2+}. The last member of this subgroup – $P2Y_{11}$ can be coupled to either $G_{q/11}$ or G_s proteins. In consequence, it is positively linked to two signaling pathways by activating either PLC or adenylate cyclase, respectively (Communi et al. 1997; Ralevic

and Burnstock 1998). $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors, phylogenetically and structurally classified to the second subgroup of P2Y receptors, are all coupled to G_i protein and their stimulation negatively affects adenylate cyclase and decreases cAMP level (Abbracchio et al. 2003; Moore et al. 2003) (Fig. 3.1). Thus, a slower response of P2Y receptors to agonists than that of P2X is a result of the involvement of the second messenger systems.

From a pharmacological point of view, the P2Y receptor family is characterized by different sensitivities to adenine and/or uridine nucleotides (see Table 3.1). It has been found that ADP is the most potent natural agonist of $P2Y_1$ receptors, while UTP and UDP are not effective (Leon et al. 1997). However, the stable analog of ADP, 2-methylthio ADP (2MeSADP), is even a more potent $P2Y_1$ agonist. $P2Y_1$ is blocked by suramin, pyridoxal-phosphate-6-azophenyl-2′,4′ disulfonic acid (PPADS), MRS2179 and MRS2269. The two last compounds were identified as selective antagonists of this receptor (Burnstock 2007) (Table 3.1). $P2Y_2$ responds to both ATP and UTP with approximately equal potency and it is insensitive to or only weakly activated by ADP or 2MeSADP (Nicholas et al. 1996). It is worth to add that two rodent receptors: $P2Y_2$ and $P2Y_4$ are equipotently activated by ATP and UTP. They can be distinguished by their proper antagonists, since suramin blocks $P2Y_2$ while Reactive blue 2 blocks $P2Y_4$ receptors (Burnstock 2007). Contrary to rat, the human $P2Y_4$ receptor is highly more selective to UTP than ATP (Nicholas et al. 1996). Furthermore, the $P2Y_6$ receptor is UDP-sensitive and is activated weakly, if at all, by UTP, ATP or ADP (Nicholas et al. 1996). The $P2Y_{11}$ receptor was cloned from human placenta and has been shown to be activated by ATP and ADP but not by UTP or UDP (Communi et al. 1997). $P2Y_{12}$ and $P2Y_{13}$ receptors are phylogenetically and structurally related (Communi et al. 2001). They are selective for ADP and 2MeSADP and blocked by ARC- compounds. The $P2Y_{13}$ receptor can be distinguished from $P2Y_{12}$ by the use of PPADS, which acts as an antagonist of $P2Y_{13}$ (but not of $P2Y_{12}$), or by the use of p^1,p^4-di(adenosine-5′)tetraphosphate (Ap_4A), which acts as an agonist of $P2Y_{12}$ and as an antagonist of the $P2Y_{13}$ receptor (Claes et al. 2001). The recently cloned $P2Y_{14}$ receptor is activated by UDP-glucose, UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetyl-glucosamine, while no significant responses are detected when ATP, ADP or UTP are used (Abbracchio et al. 2003; Harden et al. 2010).

As mentioned before (see Sect. 3.2.2), all receptors belonging to the $P2Y_{12}$-like subfamily are exclusively coupled to $G_{i/o}$ protein and decrease cAMP level in cells (Harden et al. 2010). However, it has also been suggested that the $P2Y_{14}$ receptor coupled to $G_{i/o}$ protein can activate PLCβ *via* β/γ subunits of this protein (Bianco et al. 2005). A similar activation of PLCβ *via* $G_{i/o}$ protein coupling might occur in the case of other P2Y receptors and among them $P2Y_2$ (Burnstock 2007).

Interestingly, for the last several years a couple of reports appeared describing a change in the downstream pathways activated by nucleotide receptors after binding integrins. It was shown that the arginine-glycine-aspartic acid (RGD) domain is responsible for selectively attaching $\alpha_V\beta_3/\beta_5$ integrins to $P2Y_2$ receptors in human astrocytoma cells (Erb et al. 2001). Thus, UTP is able to stimulate G_0 and G_{12} proteins which lead to the activation of small GTPases: Rac and Rho – proteins crucial for actin cytoskeletal rearrangements required for chemotaxis (Bagchi et al. 2005; Liao et al. 2007). Moreover, in primary rat astrocytes, UTP-stimulated cell migration was inhibited by anti α_V integrin antibodies (Wang et al. 2005). All those data suggest that in regions of strong substratum adhesion, $P2Y_2$ receptors may transduct signals through subunits other than G_q, but also that $P2Y_2$-associated pathways may be important factors regulating migration-dependent events like astrogliosis in brain disorders. The above issues will be precisely described in Chap. 6 of this book.

P2Y receptors are widely distributed in nearly all human and rodent tissues. Several neuronal and glial cell lines have been derived from human or rodent brain (see Chap. 1 and, for review, see Sak and Illes 2005).

It has been suggested that in CNS, P2Y receptors may take a part in many basic functions, such as cellular metabolism, proliferation and differentiation, both under physiological and pathological conditions (Abbracchio and Verderio 2006; Di Virgilio et al. 2001). In rat cortical astrocytes,

Table 3.1 The main properties of P2Y receptors

Subtype	Chromosomal location of the gene (*H. sapiens*)	Number of amino acids	Agonists activity	Antagonists	The main activated pathways
$P2Y_1$	3q25.2	373	2-MeSADP > 2-MeSATP = ADP > ATP	MRS 2279, MRS 2179, PPADS, suramin	$G_{q/11} \Rightarrow Ca^{2+}\uparrow$
$P2Y_2$	11q13.5-q14.1	377	UTP = ATP	suramin	$G_{q/11} \Rightarrow Ca^{2+}\uparrow$
$P2Y_4$	Xq13	365	UTP ≥ ATP	Reactive blue 2, PPADS	$G_{q/11} \Rightarrow Ca^{2+}\uparrow$
$P2Y_6$	11q13.5	328	UDP > UTP >> ATP	Reactive blue 2, PPADS, suramin	$G_{q/11} \Rightarrow Ca^{2+}\uparrow$
$P2Y_{11}$	19p13.2	374	AR-C67085MX > BzATP ≥ ATPγS > ATP	suramin, reactive blue 2,	$G_{q/11} \Rightarrow Ca^{2+}\uparrow$ $G_s \Rightarrow cAMP\uparrow$
$P2Y_{12}$	3q24-q25	342	ADP = 2-MeSADP	AR-C67085MX, cangrelor	$G_i \Rightarrow cAMP\downarrow$
$P2Y_{13}$	3q24	354*	ADP = 2-MeSADP >> ATP & 2-MeSATP	AR-C67085MX, cangrelor	$G_i \Rightarrow cAMP\downarrow$
$P2Y_{14}$	3q24-q25.1	338	UDP-glucose = UDP-galactose	–	$G_{i/0} \Rightarrow cAMP\downarrow; Ca^{2+}\uparrow$

Designations

2-MeSADP, 2-Methylthioadenosine- 5′- O- diphosphate; 2-MeSATP, 2- Methylthioadenosine- 5′- O-triphosphate; ATPγS, adenosine 5′-0(3-thiotriphosphate); AR-C67085MX, 2-Propylthio-D-β,γ-dichloromethylene-ATP; cangrelor, (known also as: AR-C69931MX) – [(2R,3S,4R,5R)-3,4-dihydroxy-5-[6-(2-methylsulfanylethylamino)-2-(3,3,3-trifluoropropylsulfanyl)purin-9-yl]oxolan-2-yl]methyl dihydrogen phosphate; BzATP, 2′,3′-*O*-(4-benzoylbenzoyl)-adenosine 5′-triphosphate; MRS 2179, 3′-Adenylic acid,2′-deoxy-N-methyl-, 5′-(dihydrogen phosphate); MRS 2279, (1R,2S,4S,5S)-1-[(phosphato)methyl]-4-(2- chloro-6-aminopurin-9-yl) bicyclo [3.1.0]-hexane-2- phosphate; PPADS, Pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid

↑ increase

↓ decrease

*49 % identity in amino acid sequence with $P2Y_{12}$ receptor

Fumagalli et al. described the expression of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{14}$ receptors and suggested their participation in astrocyte-to-astrocyte communications (Fumagalli et al. 2003). This phenomenon occurs due to the release of ATP, which may act as an autocrine and paracrine messenger initiating purinergic signaling processes. A similar role of these receptors in communication between astrocytes and neurons was also reported (Bezzi and Volterra 2001).

The expression of P2Y receptors was examined in mouse microglial cell line N9 and in primary microglia derived from rat brain, cultivated either in resting conditions or exposed to bacterial lipopolysacharide (LPS) (Bianco et al. 2005). Long-term exposure to LPS resulted in the increase of $P2Y_6$ and $P2Y_{14}$ receptors activity. This was explained as a part of a protective mechanism that minimizes sensitivity of microglia to external cell death-inducing signals (Bianco et al. 2005). These data are in agreement with the supposition that the $P2Y_{14}$ receptor may play an important neuroimmune function (Abbracchio et al. 2003). Similarly, in 1321N1 astrocytoma cells, activation of $P2Y_6$ by UDP protects cells against TNF-α induced apoptosis (Kim et al. 2003). Moreover, $P2Y_6$ mRNA expression appeared only after implantation of glioma C6 cells into rat brain and further growth in culture (*ex vivo* model), while in C6 control cell line these receptors were not detected. In general, glioma C6 cells express multiple P2Y receptor subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ (Barańska et al. 2004; Braganhol et al. 2009; Czajkowski and Barańska 2002; Krzemiński et al. 2008; Van Kolen and Slegers 2006b).

In rat native cerebellar astrocytes, Carrasquero et al. described expression of $P2Y_1$ and $P2Y_{13}$ receptors, while $P2Y_{12}$ was not detected (Carrasquero et al. 2005). The authors suggested that in these cells $P2Y_{13}$ substitutes for $P2Y_{12}$ and cooperates with $P2Y_1$, similarly as it is observed in platelets, where $P2Y_1$ and $P2Y_{12}$ take part in ADP-induced platelet aggregation (Gachet 2006; Hollopeter et al. 2001). The $P2Y_{13}$ receptor was found to be highly expressed not only in human and mouse brain tissue but also in rat primary astrocytes, where it was proposed to participate in reactive astrogliosis (Fumagalli et al. 2004). In glioma C6 cells, the $P2Y_{12}$ receptor is highly expressed and functionally active. Moreover, its involvement in signaling processes is often opposite to that of the $P2Y_1$ receptor (Baranska et al. 2004; Krzemiński et al. 2007).

The expression and functional activity of $P2Y_1$ and $P2Y_{12}$ receptors in glioma C6 cells and the cross-talk between them will be discussed in further sections of this Chapter.

3.3 $P2Y_1$, $P2Y_2$ and $P2Y_{12}$ Receptor Expression and Functionality in Glioma C6 Cells

In the 1990s of the twentieth century, extracellular ATP was shown to generate IP_3 formation in glioma C6 cells, indicating the presence of purinergic receptors coupled to phosphoinositide turnover and hydrolysis (Hirano et al. 1991; Kitanaka et al. 1992; Lin and Chuang 1993, 1994).

While the presence of ATP-responding, PLC-activated $P2Y_2$ receptors in this cell line was generally accepted, the presence of $P2Y_1$ receptors that stimulate PLC activity was a subject of a serious debate. The studies concerning accumulation of IP_3, both in C6-2B and in C6 cells, suggested the presence of $P2Y_1$-like receptors affecting not IP_3 formation but inhibition of adenylate cyclase. Moreover, these receptors were not blocked by the $P2Y_1$ receptor antagonist, PPADS (Boarder and Hourani 1998; Schachter et al. 1996, 1997). Therefore, the existence of two different ADP-responding receptors has been proposed: one ($P2Y_1$), coupled to PLC and blocked by PPADS and another one, termed P2Y-C6 or $P2Y_{AC}$, present in glioma C6 cells and negatively coupled to adenylate cyclase (Claes et al. 2001; Grobben et al. 2001). On the other hand, Webb and others reported that the cloned rat $P2Y_1$ receptor DNA and that derived from glioma C6 cells were 100 % identical in the entire coding region (Webb et al. 1996). Thus, the authors suggested that in different cell types the same $P2Y_1$ receptor might either activate PLC or inhibit adenylate cyclase.

These confusing data were finally clarified by experiments performed in our laboratory in which the activity of $P2Y_1$ receptors was estimated by their ability to increase $[Ca^{2+}]_i$. We have previously shown that glioma C6 cells belong to the type of non-excitable cells (Barańska et al. 1995). In such cells, the voltage-dependent Ca^{2+} channels are absent and the biphasic, capacitative Ca^{2+} signaling is mediated by the inositol phosphate system (Berridge 1995; Putney and Bird 1993; see also Chap. 4). In this process, the initial rise of $[Ca^{2+}]_i$ (the first phase of the cell response) results from a direct effect of IP_3 on the ER calcium stores and can be observed even in the absence of external Ca^{2+}. The second phase of the calcium signal is generated by an opening of voltage-independent plasma membrane Ca^{2+} channels and Ca^{2+} inflow into the cell as a consequence of the depletion of the ER stores. We have shown that in glioma C6 cells, ADP as well as ATP and UTP initiate a calcium response typical for non-excitable cells and compatible with the capacitative model of Ca^{2+} influx (Sabala et al. 2001). All the above mentioned nucleotides induce $[Ca^{2+}]_i$ elevation which starts with an initial peak response (the first phase) and is followed by the second phase – a long, sustained plateau (Fig. 3.1b). Moreover, soon afterwards, cells treated with ATP failed to respond to UTP and *vice versa*, whereas similar cross-desensitization between ADP and UTP, or ADP and ATP did not occur. These results indicate that ATP and UTP act on the same common subtype of P2Y receptors ($P2Y_2$), whereas ADP is recognized by another one (Sabała et al. 2001). Treatment of cells with PPADS and MRS2179, the $P2Y_1$ receptor specific antagonists, abolish ADP-induced Ca^{2+} mobilization. The $P2Y_2$ receptor is insensitive to these compounds. Furthermore, 2MeSADP, a selective agonist of high potency for the $P2Y_1$ receptor, is more efficient than ADP in increasing $[Ca^{2+}]_i$ (Czajkowski et al. 2002; Sabała et al. 2001).

As it is shown in Fig. 3.1b, the kinetics of ADP-evoked $[Ca^{2+}]_i$ changes differs from those generated by ATP and UTP. The response is more transient and quickly returns to the basal level. Therefore, one could expect that ADP-evoked IP_3 accumulation was also transient and too short to be estimated. This could be a reason for the former belief that in glioma C6 cells, ADP-responding receptor stimulation did not lead to PLC activation. However, using a direct visualization of GFP coupled PLCδ-PH domain of PIP_2 in C6 cells, we revealed that after stimulation with ADP, the PIP_2-bound GFP fluorescence was reduced at the cell surface, indicating the breakdown of PIP_2 by activated PLC (Fig. 3.1a) (Czajkowski et al. 2002).

In contrast to PLC activation, ADP-evoked adenylate cyclase inhibition has been documented for a long time (Boyer et al. 1993). Thus, in glioma C6 cells ADP has two activities: it stimulates PLC and inhibits adenylate cyclase. The latter activity can be shown by inhibition of the isoproterenol-elicited cAMP accumulation by 80 % in the presence of ADP (Fig. 3.1c). This effect is reversed by the treatment of cells with pertussis toxin (PTX), indicating G_i protein involvement, and is insensitive to PPADS (Sabała et al. 2001).

Knockdown of the $P2Y_1$ receptor with antisense oligonucleotides, as well as treatment of cells with MRS2179 or PPADS, caused loss of receptor-mediated PLC activity but were without effect on ADP- induced adenylate cyclase inhibition. All of these indicate that there exist two different receptors in glioma C6 cells, both responding to ADP (Czajkowski et al. 2002). At the same time, a P2Y receptor subtype, previously called $P2T_{AC}$, that is coupled to the inhibition of adenylate cyclase in rat blood platelets, was cloned and designated as $P2Y_{12}$ (Hollopeter et al. 2001; Savi et al. 2001). The presence of this receptor was also reported in glioma C6-2B cells (Jin et al. 2001). Its identity and the presence in glioma C6 cells, as well as the presence of $P2Y_1$ and $P2Y_2$ receptors, was verified and confirmed by polymerase chain reaction using reverse-transcribed total RNA (RT-PCR) and Western blot analysis (Barańska et al. 2004; Czajkowski et al. 2002, 2004; Krzemiński et al. 2007; Sabała et al. 2001) (Fig. 3.2).

Thus, in glioma C6 cells the $P2Y_1$, $P2Y_2$ and $P2Y_{12}$ receptors are expressed. ATP and UTP act as agonists on $P2Y_2$, whereas ADP stimulates both $P2Y_1$ and $P2Y_{12}$ receptors. The molecular identity, pharmacological analysis and the typical biphasic Ca^{2+} response showed that $P2Y_2$ and $P2Y_1$ are linked to PLC and Ca^{2+} release, whereas $P2Y_{12}$ is negatively coupled to adenylate cyclase through G_i protein.

In conclusion, it is worth to add that one of the most characteristic features of glioma C6 cells is the massive expression of the $P2Y_{12}$ receptor, long known to exist in this cell line and previously

denominated as P2Y-C6 or $P2Y_{AC}$. High expression of this receptor leads to a very low constitutive basal level of cAMP (see Fig. 3.1c). The other characteristic feature is the instability of the $P2Y_1$ receptor expression. The lack of activity of the $P2Y_1$ receptor reported previously (Grobben et al. 2001; Schachter et al. 1997) might be explained not only by the transient and very short stimulation of PLC and IP_3 accumulation but also, and most likely, by conditions of cell culturing and handling. It has been recently shown that the long-term absence of serum in the C6 cell culture medium specifically affects the $P2Y_1$ receptor, strongly decreasing its expression and, simultaneously, its activity (Barańska et al. 2004; Czajkowski et al. 2004; Krzemiński et al. 2007 and see Chap. 4). The effect of serum withdrawal on $P2Y_1$ and $P2Y_{12}$ receptor expression is presented in detail in the next Section.

3.4 Serum Withdrawal

3.4.1 Effect on $P2Y_1$ and $P2Y_{12}$ Receptor Expression

It is well established that proliferation of cells is induced by numerous substances present in animal serum such as growth factors, cytokines or bioactive lipids. Among them, an important serum component is lysophosphatidic acid, bound to albumin and carried in the blood stream (Jalink et al. 1994; Kranenburg and Moolenaar 2001). Therefore, media used for cells cultivation are usually supplemented with sera originating from various sources. However, it is known that for some *in vitro* studies, such as mitogenic signaling, it is better to use withdrawal cells (Grobben et al. 2001; Tu et al. 2000). Such proceeding should also be applied for glioma C6 cells in the case of mitogen-activated protein kinase studies, including extracellular signal-regulated kinases 1/2 (ERK1/2). In cells cultivated in the presence of serum the basal activity of ERK1/2 is so high that nucleotides, ADP and UTP, are unable to induce additional stimulation of ERK1/2 activity (Barańska et al. 2004; Czajkowski et al. 2004).

Sometimes, differences in cell culture conditions may be misleading. The group of Slegers was unable to detect activation of PLC and calcium release after ADP stimulation in glioma C6 cells grown in chemically defined serum-free medium. In consequence, the authors suggested the lack of $P2Y_1$ receptor expression in those cells (Grobben et al. 2001). In contrast to the results of Slegers' group, studies performed on cells growing in the medium supplemented with serum revealed the presence and functional activity of the classic $P2Y_1$ receptors (Czajkowski et al. 2002; Sabała et al. 2001). These data show that variations in the composition of the culture medium, such as the presence or absence of serum, may have important implications. Therefore, comparison of the effects of these two cell culture conditions seemed to be essential (Czajkowski et al. 2004; Krzemiński et al. 2007).

Figure 3.2a shows the mRNA level of $P2Y_1$, $P2Y_2$ and $P2Y_{12}$ receptors in glioma C6 cells, either serum-deprived or cultured in the presence of 10 % fetal bovine serum. In cells starved for 48 h in the serum-free medium, the level of $P2Y_1$ mRNA strongly decreased, whereas $P2Y_{12}$ mRNA expression slightly increased. When C6 cells were cultured for 98 h in chemically defined serum-free medium, as described by Grobben et al. (Grobben et al. 2001), the same effect was observed (Czajkowski et al. 2004) (not shown in Fig. 3.2). In contrast, serum deprivation was without any effect on $P2Y_2$ mRNA expression (Fig. 3.2a). A similar pattern of changes was demonstrated for proteins (Krzemiński et al. 2007). Figure 3.2b shows the level of $P2Y_1$ and $P2Y_{12}$ receptor protein in cells cultivated for 24, 48, 72 and 98 h in the serum-free medium. The level of the $P2Y_1$ receptor gradually decreased and was very low after 72 h, and particularly after 96 h, while the level of $P2Y_{12}$ increased. However, even after 96 h of serum deprivation, when the medium was replaced by that containing serum, the level of the $P2Y_1$ receptor could be restored (Fig. 3.2c).

The interrelation between $P2Y_1$ and $P2Y_{12}$ expression led to the hypothesis, that increased level of $P2Y_{12}$ receptors, observed during long-time serum starvation, might be a consequence of $P2Y_1$ decrease. Up to now, there are no literature data about the regulation of transcription of $P2Y_1$ and

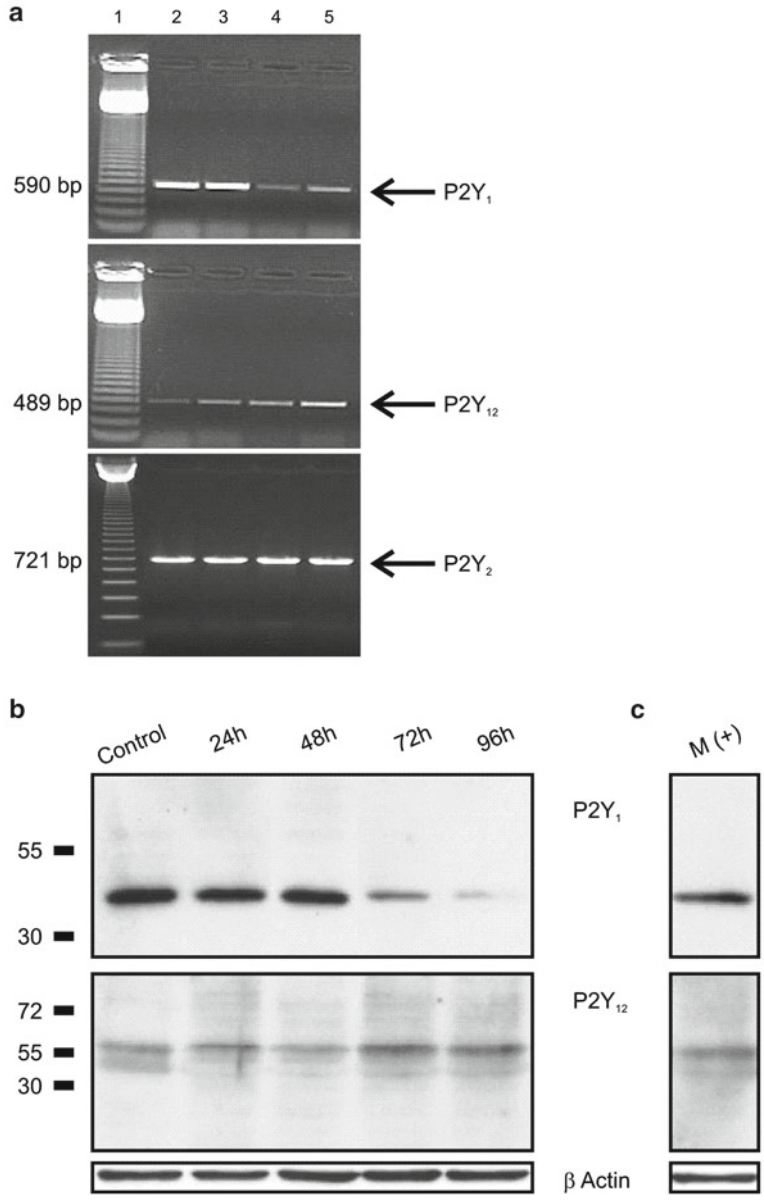


Fig. 3.2 Effect of serum deprivation on mRNA and protein levels of nucleotide receptors in glioma C6 cells. (**a**) Detection of $P2Y_1$, $P2Y_2$ and $P2Y_{12}$ mRNA by RT-PCR reaction. Amplification products of 590, 489 and 721 bp correspond to $P2Y_1$, $P2Y_{12}$ and $P2Y_2$ mRNAs, respectively. Lane 1, 123 DNA ladder; lanes 2 and 3, cells grown in the medium supplemented with 10 % serum; lanes 4 and 5, cells after 48 h serum deprivation. (**b**) Changes in the protein level of $P2Y_1$ and $P2Y_{12}$ receptors detected using Western blot in non-starved cells (control) and cells cultivated without serum for 24, 48, 72 and 96 h, respectively. (**c**). Cells cultivated in serum-free medium for 96 h followed by 24 h recovery in fresh medium with serum (M+) (Reproduced from Barańska et al. (2004) (**a**) with permission from Elsevier and from Krzemiński et al. 2007 (**b** and **c**) with permission from John Wiley and Sons)

$P2Y_{12}$ genes. Furthermore, rat genome analysis in the Ensembl database showed no essential differences between $P2Y_1$ and $P2Y_{12}$ gene promoters in transcription factor binding sites, which were additional arguments in favor of the hypothesis. The development and spreading of the siRNA technique encouraged its verification.

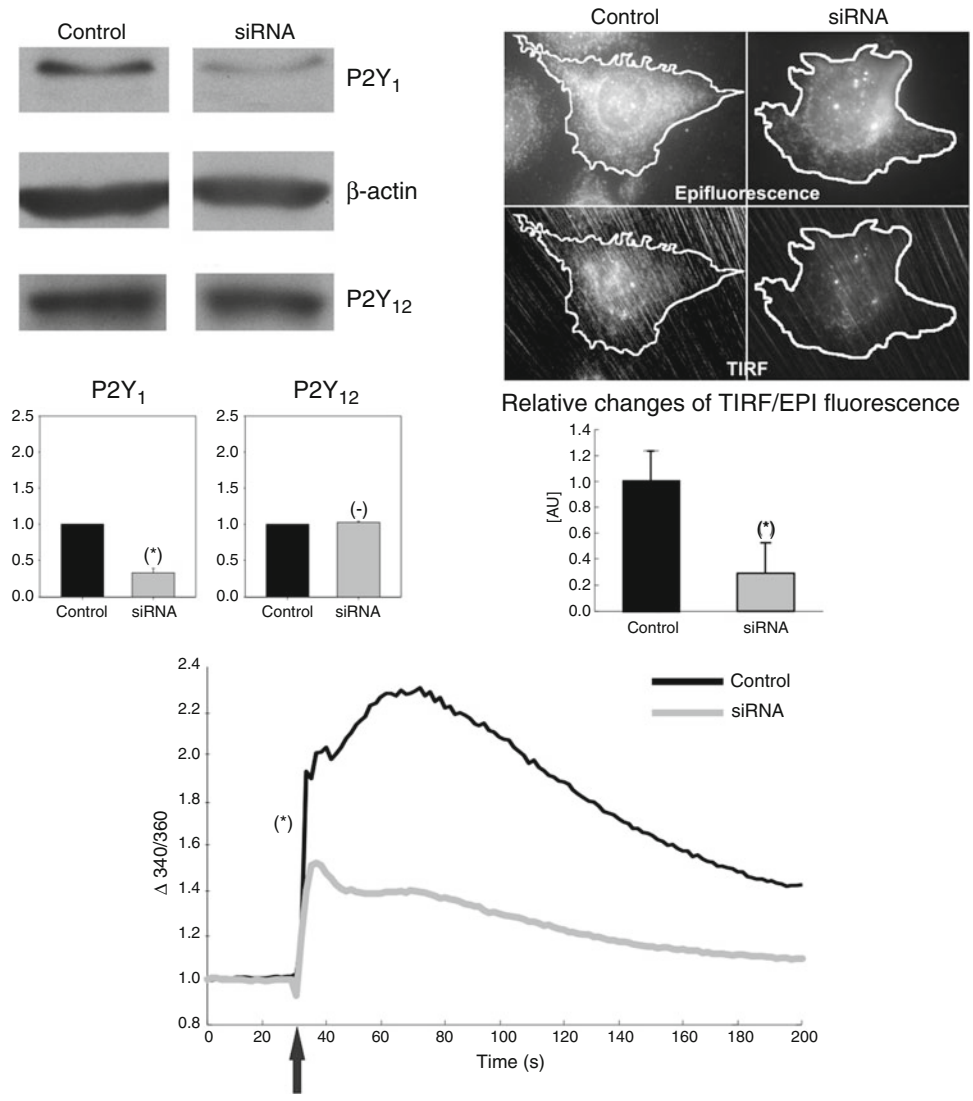


Fig. 3.3 Selective exclusion of $P2Y_1$ receptors by siRNA in glioma C6 cells. (**a**) A typical Western blot experiment. The level of each receptor protein in total protein extract was measured densitometrically and is presented with reference to a control protein (β-actin) (number of analyzed experiments, $N=3$). The protein level in the control is assumed as 1 arbitrary unit (AU). The analysis shows a significant decrease of $P2Y_1$ receptor protein 0.32 ± 0.07 AU ($p<0.05$) and no change in the amount of $P2Y_{12}$ receptor protein 1.02 ± 0.02 AU. (**b**) Immunocytochemical staining of $P2Y_1$ receptors visualized in epifluorescence (*upper pictures*) and in Total Internal Reflection Fluorescence Microscopy (TIRF) module (*lower pictures*). The relative ratio between $P2Y_1$ receptors on the plasma membrane to the total amount of receptors in control cells is 1 ± 0.22 AU (number of analyzed cells, $n=14$) and after siRNA transfection it decreases to 0.29 ± 0.23 AU ($n=18$). The change is statistically significant ($p<0.05$). (**c**) Functional study of $P2Y_1$ receptors – the mean calcium response of control (*black*) and siRNA transfected cells (*grey*) evoked by 10 μM 2MeSADP as indicated by *arrow*, in buffer with 2 mM $CaCl_2$. The change was statistically significant ($p<0.001$)

The selective exclusion of the $P2Y_1$ receptor was confirmed by Western blot and TIRFM (Total Internal Reflection Fluorescence Microscopy). Both analyses showed a decreased level of $P2Y_1$ receptors in total protein extracts and in the plasma membrane, respectively (Fig. 3.3a, b). Moreover, studies of the activity of the $P2Y_1$ receptor confirmed this result (Fig. 3.3c). However, further experiments showed that decrease of the $P2Y_1$ protein level did not cause an increase of the level of $P2Y_{12}$ receptors

(Fig. 3.3a). Similarly, the inhibition of $P2Y_{12}$ expression by siRNA did not lead to the increase of $P2Y_1$ (not shown). In other words, the increased level of $P2Y_{12}$ receptors is not the consequence of inhibition of $P2Y_1$ receptor expression, but is the result of changes in cell culture medium, i.e. serum withdrawal (unpublished results). It can be deemed that the lack of factor/factors present in serum causes changes in expression of both these receptors independently. Nevertheless, the change in expression of $P2Y_1$ and $P2Y_{12}$ during long-term serum starvation is very strong, comparable with a selective experimental blockade by siRNA. Hence, long-term serum starvation is a good model for functional studies of both these receptors.

In conclusion, the long-term serum deprived glioma C6 cells are characterized by very low expression of the $P2Y_1$ receptor. Under such conditions, the $P2Y_{12}$ receptor strongly predominates and is primarily responsible for the ADP-evoked signal transduction. Consequently, the decrease in $P2Y_1$ mRNA and protein expression is reflected by the distinctly lower ADP-evoked calcium response when compared with cells incubated in the presence of serum (Barańska et al. 2004).

In the very instructive review article Sak and Illes reported that in neuronal cancer cell lines of both human and rodent origin, the $P2Y_1$ subtype of P2Y receptors appears to be absent (Sak and Illes 2005). This phenomenon seems to be a general property of neuronal malignant cells. In contrast, in healthy neuronal cells, the $P2Y_1$ receptor predominates (Illes and Alexandre Ribeiro 2004). In an inhospitable environment created by prolonged serum starvation, glioma C6 cells demonstrate similar malignant-like behavior.

3.4.2 Effect on C6 Cell Morphology, Growth and Differentiation

Glioma C6 cells normally have an irregular, flattened shape. They are usually characterized by a typical, bipolar, fibroblast-like morphology (see Chap. 4). However, during prolonged (up to 96 h) serum deprivation 75 % of cells gradually adopted a rounded, astrocyte-like shape. Nevertheless, after 96 h of serum deprivation, when the medium was replaced with one containing 10 % serum, the cells returned to their typical fibroblast-like morphology and the amount of altered cells decreased to about 25 % (Krzemiński et al. 2007).

Long-term serum withdrawal also induced cell cycle arrest. Such effect was demonstrated in many cell lines including astrocytes (Chou and Langan 2003). Tu et al. reported that in glioma C6 cells, 48 h serum deprivation induced growth arrest synchronized in the G_o/G_i phase of cell cycle and the cells minimally incorporated [^{3}H]tymidine, a marker of proliferation (Tu et al. 2000). It was shown that 96 h of serum deprivation arrested 95 % of C6 cells in the G_o/G_i phase (Krzemiński et al. 2007). Again, addition of medium containing serum almost completely restored cells to the control pattern. These data indicate that the observed alterations are not permanent and cells are still ready to be activated. Furthermore, serum deprivation did not cause a significant loss of viability or apoptotic features of glioma C6 cells (Krzemiński et al. 2007).

Similarly to the long-term (days) serum deprivation, the same morphological changes could be observed when cells were incubated only for 10 min in a medium without serum and then, still in the absence of serum, treated with agonists that increased intracellular concentration of cAMP (Oey 1975). Furthermore, Koschel and Tas showed that the treatment of glioma C6 cells with isoproterenol, an agonist of β-adrenergic receptor positively coupled to adenylate cyclase, resulted in a rapid increase in cAMP and subsequently induced a change in the cell morphology from a flat, fibroblast-like to a rounded, astrocyte-like shape (Koschel and Tas 1993). This change was completed in about 30–40 min and occurred only in the absence of serum in cell culture media. The presence of serum prevented these changes and its addition to the morphologically changed cells, restored the fibroblast-like appearance. The component of serum responsible for this effect was identified as lysophosphatidic acid (Koschel and Tas 1993). Sphingosine 1-phosphate, another bioactive lipid present in serum, was

also found to be able to reverse this change (Tas and Koschel 1998). It has been further found that lysophosphatidic acid and sphingosine 1-phosphate receptors are widely expressed in numerous cell types. They can be coupled to multiple G proteins: G_i, G_q and $G_{12/13}$ and mediate variety of responses, among them a long-term stimulation of cell proliferation (Kranenburg and Moolenaar 2001; Moolenaar 1999; van Koppen et al. 1996). In fibroblasts, lysophosphatidic acid acting on a receptor coupled with G_i protein inhibits, similarly as the $P2Y_{12}$ receptor, adenylate cyclase, and leads to a decrease of intracellular cAMP level (van Corven et al. 1989).

In glioma C6 cells growing in chemically defined serum-free medium, agents activating β-adrenergic receptor or membrane-permeable cAMP analogues stimulating adenylate cyclase activity (such as dibutyryl cAMP), induced the synthesis of glial fibrillary acidic protein (GFAP) concomitantly with growth arrest (Claes et al. 2004; Roymans et al. 2001). GFAP is widely expressed in astrocytes and is used as a marker of the induction of differentiation into an astrocyte type II (Dahl 1981; Messens and Slegers 1992). Together with changes in GFAP expression, C6 cells, more rounded than cells growing in the presence of 10 % serum (Messens and Slegers 1992), became even more astrocyte-like with a stellate shape (Claes et al. 2004). Furthermore, this cAMP-induced differentiation of C6 cells could be inhibited by activation of the $P2Y_{12}$ receptor (Claes et al. 2004).

In contrast to control conditions, long-term serum starvation, although it imposed growth arrest and astrocyte-like appearance on C6 cells, did not induce differentiation into astrocytes and oligodendrocytes. Under such conditions, expression of GFAP and NG2 proteoglycan (a marker of oligodendrocyte progenitors) was not increased either in the presence or absence of serum in the culture medium (Krzemiński et al. 2007). Taken together, these data pointed to the role of the $P2Y_{12}$ receptor in cell survival. Different expression of $P2Y_1$ and $P2Y_{12}$ receptors in serum-starved and non-starved cells seems to have an important biological significance. The inhibitory effect of $P2Y_{12}$ on adenylate cyclase activity might maintain the low intracellular cAMP level and, due to that, block cAMP-induced differentiation of C6 cells, partially compensating for the lack of serum in the culture medium.

3.4.3 *Effect on the $P2Y_{14}$ Receptor*

As it was already mentioned above, the $P2Y_{14}$ receptor responds to sugar nucleotides, such as UDP-glucose, UDP-galactose, UDP-glucoronic acid and UDP-N-acetyl-glucosamine. Phylogenetically it belongs to the group of $P2Y_{12}$ and $P2Y_{13}$ receptors (Abbracchio et al. 2003; Harden et al. 2010). $P2Y_{14}$ shares 45 % homology with the $P2Y_{12}$ receptor and, similarly to $P2Y_{12}$ and $P2Y_{13}$, appears to be coupled to PTX-sensitive $G_{i/o}$ protein. The studies on HEK 293 cells co-transfected with $P2Y_{14}$ and different chimeric G α subunits recognized and confirmed that the $P2Y_{14}$ receptor is coupled to $G_{i/o}$ proteins (Moore et al. 2003). Indeed, in murine T-lymphocytes and human neutrophils UDP-glucose induces inhibition of adenylate cyclase (Moore et al. 2003; Scrivens and Dickenson 2005, 2006). However, in rat cortical astrocytes, human monocytes and N9 microglial cells, UDP-glucose also stimulates elevation of intracellular Ca^{2+} level (Bianco et al. 2005; Fumagalli et al. 2003; Skelton et al. 2003).

Our studies showed the presence of the $P2Y_{14}$ receptor on glioma C6 cells. Western blot technique and the treatment with deglycosylation enzyme, N-glycosidase F, demonstrated that in these cells the $P2Y_{14}$ receptor exists in two forms: glycosylated, which highly predominates, and non-glycosylated one (Krzemiński et al. 2008). Binding of UDP-glucose induced two responses sensitive to PTX: an increase of calcium concentration and an inhibition of adenylate cyclase. This suggests participation of the β/γ subunits of $G_{i/o}$ protein in PLCβ activation, as it was previously proposed by Bianco et al. (2005). However, this result does not exclude the possibility that two UDP-glucose receptors exist: one coupled to PLC and another one negatively coupled to adenylate cyclase (Krzemiński et al. 2008). It is worth to add that recently UDP was found to be a potent agonist of both human and rodent $P2Y_{14}$ receptors (Carter et al. 2009; Fricks et al. 2008, 2009). Harden et al. hypothesized that $P2Y_{14}$ and

$P2Y_6$, co-existing in many tissues, might be coordinately activated by UDP, resulting in G_q-dependent activation of PLC by $P2Y_6$ and G_i-dependent inhibition of adenylate cyclase by $P2Y_{14}$, similarly as in the case of $P2Y_1$ and $P2Y_{12}$ receptors activated by ADP (Harden et al. 2010).

Since in glioma C6 cells long-term serum deprivation distinctly affects expression and activity of $P2Y_1$ and $P2Y_{12}$, the same procedure was applied for studies concerning the $P2Y_{14}$ receptor. It was found that during long-term serum deprivation expression of the glycosylated form strongly decreased, whereas that of the non-glycosylated form increased. Simultaneously, calcium response induced by UDP-glucose was reduced in serum-starved cells. Moreover, in 96 h serum-deprived cells, inhibition of isoproterenol-induced cAMP accumulation was two times higher than in non-starved cells. These data show that the glycosylated form of the $P2Y_{14}$ receptor is primarily involved in intracellular calcium mobilization, while the non-glycosylated one in adenylate cyclase inhibition (Krzemiński et al. 2008).

What seems to be the most interesting is the response of glioma C6 cells to the long-term serum deprivation. Serum starvation favors an increase in the expression of receptors, whose activation inhibits cAMP accumulation, such as $P2Y_{12}$ and the non-glycosylated form of $P2Y_{14}$. Since cAMP has been shown to inhibit growth and induce differentiation of glioma cells (Dugan et al. 1999; Kim et al. 2001), such cross-talk between $P2Y_1$ and $P2Y_{12}$ receptors and between glycosylated and non-glycosylated forms of the $P2Y_{14}$ receptor seems to be an important self-regulating process acting against cells differentiation and maintaining proliferation and survival in unfavorable conditions.

3.5 Cyclic AMP Effect on Cell Proliferation, Growth and Differentiation

Cell proliferation and survival is *inter alia* regulated by growth factors *via* receptor tyrosine kinases (RTK). Activation of these cell-surface receptors lead to phosphorylation of ERK1/2 and/or activation of PI3 kinase. These two signaling pathways may be regulated by cyclic AMP.

cAMP is a common second messenger that influences many cellular processes and gene transcription *via* protein phosphorylation (Sutherland 1972). Elevated levels of cAMP lead to activation of different cAMP targets, such as the cAMP-dependent protein kinase A (PKA), considered as the main target of cAMP in eukaryotic cells. However, Epac, the guanine exchange factor (GEF) directly activated by cAMP, was also shown to be a receptor for this compound (Bos 2003; de Rooij et al. 1998; Kopperud et al. 2003). Epac specifically activates a small G protein, Rap1, a member of the Ras-like family of small GTPases (see Fig. 3.4). Depending on PKA and Epac abundance, distribution and localization, these two agents may act independently, synergistically or in opposition to each other in regulating specific cellular functions (for more details see Cheng et al. 2008)

In various types of cells, cyclic AMP may cause different effects. It is involved in regulating various cellular features of differentiation, such as neurite outgrowth in PC-12 cells, or adipocyte formation from 3 T3-L1 fibroblasts (Cheng et al. 2008; Wan et al. 2011). In epithelial cells, hepatocytes, keratinocytes, pancreatic islet β cells or Schwann cells it stimulates proliferation. On the contrary, an increased level of cAMP in fibroblasts, smooth muscle, lymphoid cells or epithelial tumor cells diminishes cell growth (Dugan et al. 1999). These divergent effects depend on whether cAMP activates or inhibits ERK1/2, whose signaling is important for cellular proliferation, differentiation and survival.

In central nervous system-derived neurons, cAMP activates ERK1/2 thus stimulating their growth and survival (Dugan et al. 1999). In spinal motor neurons cultured *in vitro*, elevation of intracellular cAMP extended neuronal survival in serum-free medium for as much as 1 week (Hanson et al. 1998). On the opposite, in primary astrocyte and glioma C6 cells, cAMP inhibits ERK1/2 activity and simultaneously inhibits cells growth (Dugan et al. 1999; Kurino et al. 1996; Qiu et al. 2000; Wang et al. 2001). Proposed mechanisms of these processes are presented in Fig. 3.4.

Thus, in a variety of cell types multiple distinct mechanisms regulate cAMP inhibition or activation of ERK1/2 signaling (for review see Stork and Schmitt 2002). Figure 3.4 shows simplified and

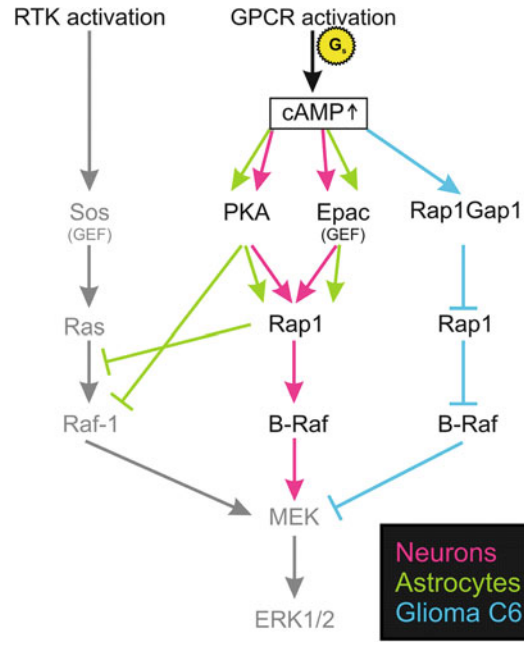


Fig. 3.4 Cyclic AMP-mediated regulation of ERK1/2 signaling in neurons (*pink*), astrocytes (*green*) and glioma C6 cells (*blue*). For details, see text

schematic models of the canonical receptor tyrosine kinase (RTK) signaling pathway (gray color) and those regulated by cAMP in neurons (pink color), astrocytes (green color) and glioma C6 cells (blue color).

RTK signaling occurs *via* a small, monomeric protein G, Ras, and a serine-threonine protein kinase, Raf-1 (Fig. 3.4, gray color). After growth factor stimulation, receptors undergo activation by dimerization and autophosphorylation on tyrosine residues. They, in turn, form a signaling complex with adaptor proteins and a GEF protein (Sos), which catalyzes guanine nucleotide exchange (GDP/GTP) on Ras. Activated Ras (Ras-GTP) recruits Raf-1 to the plasma membrane. Association with Ras results in Raf-1 activation and subsequent phosphorylation of protein kinase MEK (MAP kinase-ERK kinase), which further phosphorylates ERK1/2 both on tyrosine and threonine residues. Doubly phosphorylated ERK1/2 undergoes dimerization and translocation to the nucleus, where it serves as an activator of early response genes. In the case of receptor stimulation by the epidermal growth factor (EGF), ERK activation is an absolute requirement for cell proliferation. The role of P2Y receptors in the cross-talk between growth factor receptors and ERK1/2 activation was reviewed by Van Kolen and Slegers (2006b).

However, activation of MEK and further ERK1/2 may occur due to an alternative pathway, in which not RTK, but G-protein coupled receptors linked to G_s and responsible for intracellular cAMP accumulation take part. In neurons, elevated cAMP level activates PKA or/and Epac, which in turn activates small G protein Rap1 (Fig. 3.4, pink color). This activation occurs either due to direct cAMP binding to Epac or due to cAMP-induced phosphorylation of PKA. Epac belongs to a family of cAMP-binding nucleotide exchange factor proteins (GEFs) that catalyze GDP/GTP exchange on Rap1. Contrary to Ras, Rap1 activates the serine-threonine kinase B-Raf. Activated B-Raf phosphorylates MEK which, in turn, phosphorylates and activates ERK1/2. This pathway was described in PC12 cells (Vossler et al. 1997) and was found to occur in cells that possess the B-Raf protein. B-Raf mRNA was detected in brain, and in neurons; B-Raf seems to be the major MEK activator (Qiu et al. 2000). In contrast, astrocytes do not express this protein (Dugan et al. 1999).

In neurons, when the cAMP level is not increased, growth factors recruit Ras and activate ERK1/2. When cAMP level is elevated, Rap1 is recruited and activated by cAMP (Fig. 3.4, pink color). In astrocytes lacking B-Raf, the increased level of cAMP inhibits ERK1/2 (Fig. 3.4, green color). This

inhibition may be attributed to a direct PKA-mediated phosphorylation of Raf-1, which inhibits Ras binding and Raf-1 activity. It is also suggested that Rap1 antagonizes Ras by interfering in Ras-effectors function. Activated Rap1 (when bound to GTP) competes with Ras for Raf-1 binding and thereby blocks Ras-induced Raf-1 activation (Fig. 3.4, green color) (Dugan et al. 1999; Wang et al. 2001). These observations led to the hypothesis that an increased cAMP level stimulates ERK1/2 and induces proliferation in B-Raf expressing cells, but inhibits this cascade in B-Raf negative cells (Dugan et al. 1999).

In glioma C6 cells, the increased level of cAMP also inhibits ERK1/2 activity and cell growth. Dugan et al. suggested that in these cells, the growth-inhibitory effect of cAMP is mediated similarly as in astrocytes (Dugan et al. 1999). However, Wang et al. detected the B-Raf protein in C6 glioma cells and found that, in contrast to astrocytes, cAMP inhibits ERK1/2 by inhibiting Rap1 (Fig. 3.4, blue color) (Wang et al. 2001). Consequently, neither PKA nor Epac were involved in the cAMP-dependent inactivation of ERK1/2. The authors suggested that in C6 cells the cAMP-dependent Rap1 and ERK1/2 inhibition occurred due to Rap1Gap1 activity modulation. Gap1 (GTPase-activating protein 1) is regulated by G_i protein α subunit, which also inhibits adenylate cyclase activity (Zwartkruis and Bos 1999). Thus, an increased level of cAMP leads to Rap1Gap1 stimulation, resulting in Rap1 inhibition. Since the B-Raf protein was detected, Wang et al. proposed that cAMP inhibits ERK1/2 by inhibiting the Rap1/B-Raf/MEK/ERK signaling cascade (Wang et al. 2001). Therefore, the mechanism of this process seems to be different from the one suggested for astrocytes. However, another possibility is a direct cAMP inhibition of some GEF-Rap1 proteins, resulting in Rap1 inhibition (Wang et al. 2001).

There are, nevertheless, several examples where cAMP can inhibit cell proliferation without inhibiting ERK1/2 activity (McKenzie and Pouyssegur 1996). In embryonic fibroblast cells, Ras-1 gene removal does not affect ERK activation, although further development is blocked due to increased apoptosis. Moreover, cAMP inhibition of ERK1/2 may be time-dependent, as in PC12 cells, where cAMP blocks early activation but enhances late activation of ERK1/2 (Arslan and Fredholm 2000).

In many cells, cAMP inhibits not only ERK1/2 but also the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, also called Akt) signaling cascade. Upon RTK activation, PI3K catalytic subunit is recruited to the plasma membrane and increases the levels of phosphatidylinositol-3,4,5-trisphosphate and inositol-3,4-bisphosphate. They, in turn, induce translocation of two kinases to the plasma membrane, Akt and PDK1 (phosphoinositide (PI3K) – dependent kinase 1), by binding to the plekstrin (PH) homology domain. In the membrane, PDK1 phosphorylates and activates Akt, which further affects phosphorylation of various targets. Among them are BAD and caspase-9, two known members of the pro-apoptotic Bcl-family. Their inactivation by Akt phosphorylation results in the inhibition of apoptosis. A similar result, the rescue from apoptosis, also occurs when cells express constitutively active PI3K and ERK1/2 (Brazil and Hemmings 2001; Kim et al. 2001; Wang et al. 2001).

The inhibitory effect of cAMP on the PI3K/Akt pathway was reported in Swiss 3T3, HEK293, Cos, Rat2 and glioma C6 cells (Wang et al. 2001). In the latter cell line, inhibition of Rap1 by the Rap1Gap1 protein causes a decrease in Akt phosphorylation, suggesting that cAMP influences the Akt pathway similarly to the ERK1/2 pathway, *via* inhibition of Rap1 activity. According to Wang et al. Akt inhibition is a consequence of PI3K inhibition (Wang et al. 2001). Kim et al. explained it showing that cAMP attenuates the PI3K/Akt pathway by decreasing the lipid kinase activity of PI3K and, due to that, the level of phosphatidylinositol-3,4,5-trisphosphate (Kim et al. 2001). In consequence, PDK1 translocation to the membrane is blocked and therefore Akt cannot be phosphorylated and activated.

As mentioned before, an increased level of intracellular cAMP results in inhibition of Akt activity. It leads to growth arrest and differentiation of glioma C6 cells into type II astrocytes, accompanied by induction of GFAP synthesis and changes in cell morphology (Aerts et al. 2011; Claes et al. 2004; Van Kolen and Slegers 2004). In addition, Aerts et al. reported that these cAMP-mediated effects inhibit the expression of ecto-nucleotide pyrophosphatase/phosphodiesterase 1, an ecto-enzyme bound to the cell-surface, which regulates short-term and long-term cellular functions in the central nervous

system (Aerts et al. 2011; Neary and Zimmermann 2009). However, these cAMP-dependent effects might be reversed by the activation of $P2Y_{12}$ receptors, negatively bound to the adenylate cyclase (Van Kolen and Slegers 2004).

Thus, high concentration of intracellular cAMP is dangerous for C6 cell metabolism. In consequence of the increased cAMP level, ERK1/2 and PI3K/Akt signaling pathways are inhibited and thus, C6 cell proliferation is stopped, growth is arrested and differentiation is induced. To prevent such effects, the maintenance of the low cAMP level in this cell line is essential. The cells use various mechanisms to block cAMP synthesis. One of them, described by Weber in rapidly growing hepatoma cells, is to steadily increase phosphodiesterase activity concurrently with a decrease in adenylate cyclase activity (Weber 2002). The other, reported by Chiono et al. is to inhibit type VI adenylate cyclase, which predominates in gliomas, by increasing $[Ca^{2+}]_i$ (Chiono et al. 1995). Glioma C6 cells are characterized by a very low constitutive level of cAMP (see Fig. 3.1c), which may be caused by the high activity of $P2Y_{12}$ receptors. Accordingly, during serum deprivation $P2Y_{12}$ expression is preferentially increased, most probably as the result of the same, self-regulating mechanism maintaining a low level of intracellular cAMP.

3.6 Effect of Extracellular Nucleotides on ERK1/2 and PI3K/Akt Activity: $P2Y_1$/ $P2Y_{12}$ Cross-Talk

The effect of the $P2Y_2$ receptor on ERK1/2 activity in glioma C6 cells was examined by Tu et al. (2000). ATP- and UTP-induced stimulation of this receptor resulted in an increase in [^{3}H]tymidine incorporation and p42/p44 ERK1/2 phosphorylation. $P2Y_2$-mediated stimulation of proliferation occurred through the Ras/Raf-1/MEK/ERK signaling cascade, which was described in details in the previous Section. The pathway was modulated by PLC activation, enhanced activity of protein kinase C and Ca^{2+} release. Thus, it could be concluded that the positive effect of ATP and UTP on C6 cell proliferation and DNA synthesis involved a cross-talk between $G\alpha_q$-coupled $P2Y_2$ receptors signaling and the receptor tyrosine kinase–dependent cascade (Tu et al. 2000). The mechanism by which the $P2Y_1$ receptor stimulates ERK1/2, most probably involves a PLC-dependent pathway (Czajkowski et al. 2004), similar to that described by Tu et al. for the $P2Y_2$ receptor (Tu et al. 2000).

However, the other data showed that in transiently transfected HEK 293 cells, free β/γ subunits of heterotrimeric G_i protein could also stimulate proliferation *via* the Ras/C-Raf/MEK/ERK pathway (Ito et al. 1995). Moreover, in contrast to Tu et al. and Wang et al. (Tu et al. 2000; Wang et al. 2001), who proposed that activation of ERK1/2 in glioma C6 cells might occur through Ras/Raf-1/MEK or Rap1/B-Raf/MEK, respectively (see Sect. 3.5), the group of Slegers suggested an alternative signaling cascade in the same cell line (Grobben et al. 2001). It involved another small G protein, a monomeric GTPase, RhoA, and the signaling pathway through RhoA/PCK/Raf/MEK/ERK was proposed (Grobben et al. 2001). It was further shown that an atypical PKCζ was involved in the RhoA-dependent mitogenic signaling and the proliferation was enhanced by $P2Y_{AC}$ ($P2Y_{12}$) receptor activation. It occurred through $G\alpha_i$ (but not β/γ subunits)-dependent, RhoA-dependent/PKCζ/MEK/ERK pathway (Van Kolen and Slegers 2006a). On the other hand, Soulet et al. presented evidence that the stimulated human $P2Y_{12}$ receptor, stably expressed in Chinese hamster ovary cells, activated two major signaling mechanisms: dependent and independent on G_i protein (Soulet et al. 2004). The PTX-sensitive mechanism, in which G_i protein was involved, led to cell proliferation *via* activation of both the ERK1/2 and PI3K/Akt kinase signaling. The other, PTX-insensitive, independent of G_i, occurred *via* the activation of RhoA and Rho-kinase, resulting in actin cytoskeleton reorganization, but not in cell proliferation. The authors suggested that in the latter mechanism the $G_{12/13}$ heterotrimeric protein might be involved (Soulet et al. 2004). Thus, the above data concerning mechanisms of $P2Y_{12}$-G_i protein-coupled-receptor signaling inducing ERK1/2 activation are rather divergent. The reason for

that may be different cell lines used in experiments, different subclones of these lines formed during different passages, or various cell culture conditions. Nevertheless, the experiments concerning the activity of $P2Y_{12}$ receptors are usually performed using ADP or 2MeSADP as agonists. However, the above-mentioned compounds act not only on G_i-dependent $P2Y_{12}$, but also on G_q-dependent $P2Y_1$ receptors. Therefore, the effects of stimulation of both receptors should be investigated.

As it was already mentioned (see Sect. 3.4.1), glioma C6 cells cultivated in the presence of serum are characterized by a high constitutive activity of ERK1/2 (Barańska et al. 2004; Czajkowski et al. 2004). Similar high basal activity of PI3K/Akt is a characteristic feature of gliomas and other tumor cells (Furnari et al. 1998). The latter fact is usually explained as a result of a loss or a very low level of a lipid phosphatase (PTEN). This phosphatase has an activity antagonistic to that of PI3K since it dephosphorylates the 3′ position of phosphoinositides (Cantley and Neel 1999; Kubiatowski et al. 2001). Therefore, investigations concerning ERK1/2 and PI3K/Akt activities are usually performed in media devoid of sera. This is also why 48 and 96 h serum deprived glioma C6 cells were used in studies on ERK1/2 and PI3K/Akt activities. Under these conditions, cells were growth-arrested and incorporated [^{3}H]tymidine, a marker of proliferation, only minimally (Czajkowski et al. 2004; Krzemiński et al. 2007; Tu et al. 2000).

Czajkowski et al. showed that in 48 h serum-starved C6 cells, both p44 ERK1 and p42 ERK2 were activated in a time-dependent manner in response to ADP and 2MeSADP (Czajkowski et al. 2004). ERK phosphorylation was reduced by removal of the extracellular Ca^{2+} and by specific antagonists of $P2Y_1$ and $P2Y_{12}$ receptors. The inhibitory effect of MRS2179, a $P2Y_1$ receptor antagonist, on ADP-stimulated ERK1/2 activity was approximately 40 %. ARC69931MX, a $P2Y_{12}$ receptor selective antagonist, inhibited ADP-induced ERK1/2 phosphorylation by 85 %. Moreover, the effects of both antagonists were additive (Czajkowski et al. 2004). The same effects were observed for the cells cultivated for 96 h in serum-free medium (Krzemiński et al. 2007). Extracellular Ca^{2+} removal inhibited ADP-induced ERK1/2 activation by about 70 %, whereas the one induced by UTP (the agonist of $P2Y_2$ receptors) was completely blocked (Czajkowski et al. 2004). These data point out the participation of both $P2Y_1$ and $P2Y_{12}$ receptors in ERK1/2 activation, but also show that the effect of ADP on ERK1/2 kinase activation and cell proliferation is primarily mediated by $P2Y_{12}$ receptors.

Stimulation of PI3K activity evoked by ADP *via* $P2Y_1$ and $P2Y_{12}$ receptors was examined in C6 cells grown either in the presence or in the absence of serum in the cultivation medium (Barańska et al. 2004; Czajkowski et al. 2004). The non-starved cells were characterized by a high constitutive activity of the enzyme and their stimulation by ADP resulted in decreased PI3K activity. On the contrary, in cells cultivated for 48 h without serum, ADP evoked a gradual increase in PI3K activity. Furthermore, preincubation of 48 h serum-starved cells with MRS2179 and then, still in its presence, stimulation of the cells with ADP, markedly increased PI3K activity. It indicated that blocking of $P2Y_1$ receptors had a stimulatory effect on the kinase (Czajkowski et al. 2004). Furthermore, Krzemiński et al. demonstrated that even after 96 h cultivation in serum-free medium, glioma C6 cells were able to respond to 2MeSADP and showed enhanced Akt phosphorylation, whereas $P2Y_{12}$ antagonist, ARC69931MX, completely suppressed Akt phosphorylation (Krzemiński et al. 2007).

Figure 3.5 shows changes of constitutive ERK1/2 and Akt phosphorylation in unstimulated glioma C6 cells during long-term (for up to 96 h) serum deprivation (Krzemiński et al. 2007). As it is shown, the level of p42 and p44 ERK1/2 phosphorylation was greatly reduced. This is convergent with inhibition of cells proliferation. After 96 h of serum deprivation, the replacement of the medium for a fresh one, either serum-free (M-) or containing serum (M+), restored ERK1/2 phosphorylation. On the other hand, prolonged serum deprivation (up to 72 h) increased Akt phosphorylation and this process was stopped only after very long (96 h) serum starvation. Moreover, after this time the replacement of the medium for a fresh one, but still serum-free (M-), increased Akt phosphorylation. In contrast, fresh medium with serum (M+) had an opposite effect, as the phosphorylation of Akt was decreased.

These data show the cross-talk between PI3K/Akt activity and $P2Y_1$ and $P2Y_{12}$ receptor expression (see Sect. 3.4.1). When cells are grown in the presence of serum, $P2Y_1$ receptors signaling

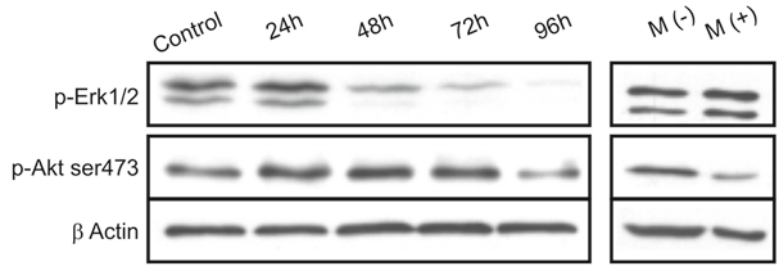


Fig. 3.5 Changes in constitutive ERK1/2 and Akt phosphorylation of unstimulated glioma C6 cells during long-term serum starvation. C6 cells were grown in the presence of 10 % fetal bovine serum (control), or were cultivated in serum-free medium for 24, 48, 72 or 96 h. (M−), cells cultivated in serum-free medium for 96 h followed by 24 h recovery in fresh medium without serum. (M+), cells cultivated in serum-free medium for 96 h followed by 24 h recovery in fresh medium with 10 % serum. The level of p42, p44 ERK1/2 and Akt phosphorylation was monitored by Western blot analysis. β-actin was used as a reference protein (Reproduced from Krzemiński et al. 2007 with permission from John Wiley and Sons)

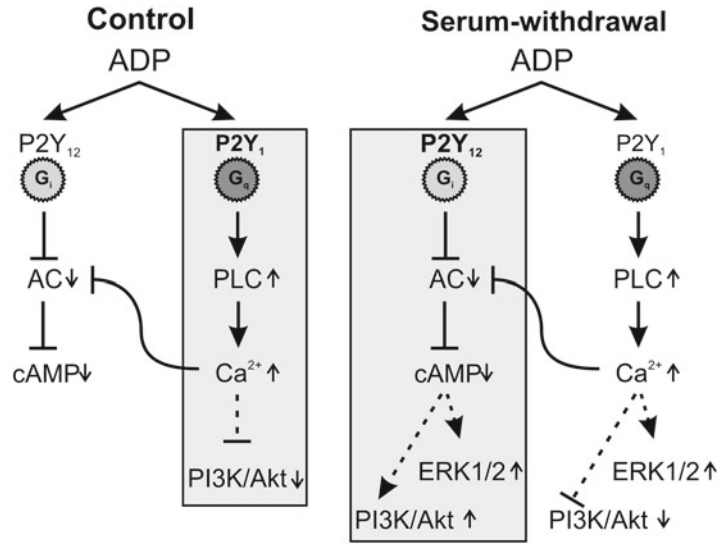


Fig. 3.6 Comparison of signal transduction pathways activated by $P2Y_1$ and $P2Y_{12}$ receptors in C6 glioma cells growing in the presence of serum (*control*) or cultivated in serum-free medium (*serum-withdrawal*). During long-term serum-starvation, the $P2Y_1$ expression strongly decreases and $P2Y_{12}$ receptor predominates. *Dashed lines* symbolize indirect stimulatory or inhibitory actions. For details, see text

predominates and inhibits P13K activity. On the other hand, when the cells are serum-deprived the expression of $P2Y_1$ receptors is strongly decreased and, in parallel, Akt phosphorylation is increased. Moreover, after 96 h of serum deprivation, when the medium is replaced for the one containing serum the protein level of $P2Y_1$ receptors increases and phosphorylation of Akt decreases again (Fig. 3.5). The inhibitory effect of the $P2Y_1$ receptor on PI3K/Akt activity is still poorly understood. On the other hand, the stimulatory effect of $P2Y_{12}$ receptors, which regulate intracellular cAMP level, on PI3K/Akt activity may occur *via* Rap1 (Aerts et al. 2011; Kim et al. 2001; Wang et al. 2001).

Thus, in C6 cells, $P2Y_{12}$ receptors are predominantly involved in ERK1/2 activation. They have a stimulatory effect on PI3K/Akt activity, whereas the effect of $P2Y_1$ receptors is inhibitory. Therefore, it seems that $P2Y_{12}$ receptors primarily contribute to glioma C6 cell growth and proliferation. The high constitutive activity of Akt, which increases further during prolonged serum starvation, seems to be also important for cell survival and enhanced invasiveness.

Signaling pathways activated by ADP-stimulation in control and serum-deprived glioma C6 cells are illustrated in Fig. 3.6.

3.7 The $P2X_7$ Receptor

As previously mentioned (see Sect. 3.2.1), the $P2X_7$ receptor is an atypical member of the P2X receptor family. It differs from other P2X receptors in that it is characterized by a relative low affinity for ATP and is activated by high, milimolar ATP concentrations. ATP binding to the $P2X_7$ opens the channel *via* a ligand-gated mechanism and evokes a fast influx of Ca^{2+} as well as Na^+ and the efflux of K^+ (North and Surprenant 2000; North 2002). Another characteristic that distinguishes $P2X_7$ from other P2X receptors is its ability to open nonspecific pores upon repeated or prolonged ATP application. These pores are responsible for the massive influx of Ca^{2+}. However, they are permeable not only to cations, but also to small molecules and high molecular weight dyes. It was reported that activation of $P2X_7$ and subsequent opening of large pores might be responsible for ATP-mediated release of amino acids in cortical astrocytes (Duan et al. 2003) as well as the release of ATP and other nucleotides in glioma C6 cells (De Vuyst et al. 2007). More recently, it was suggested that the ATP-induced ion flow through $P2X_7$ led to the activation of pannexin-1 hemichannel. In consequence, pre-interleukin-1β was prematurely processed and interleukin-1β was released from macrophages (Pelegrin and Surprenant 2006). Hemichannels are regulated by the extracellular Ca^{2+} level, they are closed at the physiological, milimolar Ca^{2+} concentration and open in response to its lowering (Srinivas et al. 2006), till apoptotic or necrotic cell death occurs (Chen and Brosnan 2006; Locovei et al. 2007).

As it was already described (see Sect. 3.2.1), activation of $P2X_7$ receptors results in reduction of membrane potential and in elevation of $[Ca^{2+}]_i$. After the opening of a receptor-channel, extracellular Ca^{2+} flows in and promotes the opening of the plasma membrane voltage-dependent calcium channels. These phenomena are usually detected by measurements of the unitary currents passing through single ion channels (patch-clamp) and measurements of $[Ca^{2+}]_i$ (North and Surprenant 2000). It is worth to add that activation of $P2X_7$ by a high, milimolar ATP concentration raises problems. First, 1 mM ATP is so acidic that it can change pH of the extracellular medium. That is why $P2X_7$ receptors are extensively activated by its analog, BzATP, which is 10–30 times more potent than ATP. The other problem is connected with the fact that BzATP is not a selective agonist of $P2X_7$, as at the same micromolar concentration it activates other P2X receptors (Anderson and Nedergaard 2006; North and Surprenant 2000; North 2002). Moreover, there is also evidence that BzATP may activate P2Y metabotropic receptors (Carrasquero et al. 2009; Fischer et al. 2009; Supłat-Wypych et al. 2010; Wildman et al. 2003). Glioma C6 cells and astrocytes are examples of such discrepancies (see below).

As mentioned in Sect. 3.2.1, $P2X_7$ receptors are widely expressed in neuronal cells. In cortical astrocytes Fumagali et al. and Bianco et al. provided evidence that $P2X_7$ receptors are functionally active (Bianco et al. 2009; Fumagalli et al. 2003). Nevertheless, using the same cultured cortical astroglia, Fischer et al. came to a different conclusion (Fischer et al. 2009). The latter authors found that BzATP caused $[Ca^{2+}]_i$ increase not only in the presence, but also in the absence of extracellular Ca^{2+}, which suggests that it activates both ionotropic and metabotropic receptors. Moreover, Carrasquero et al., measuring calcium responses in rat cerebellar astrocytes, proposed that both $P2Y_{13}$ and $P2X_7$ receptors were activated by BzATP (Carrasquero et al. 2009). However, the authors suggested that the first transient phase of calcium response was induced by the $P2Y_{13}$ receptor, whereas the second, sustained response was generated by $P2X_7$. This result seemed to be controversial since one of the main criteria to distinguish ionotropic from metabotropic receptors is the time course of the response. For ionotropic receptors, it occurs within milliseconds while the metabotropic ones respond within minutes (North and Surprenant 2000; Ralevic and Burnstock 1998).

In glioma C6 cells, neither Braganhol et al., nor De Vuyst et al., found $P2X_7$ mRNA or protein (Braganhol et al. 2009; De Vuyst et al. 2007). However, Wei et al. suggested the presence of functionally active $P2X_7$ receptors in these cells (Wei et al. 2008). This assumption was based on the fact that BzATP led to the increase of $[Ca^{2+}]_i$ and that the response was blocked by periodate-oxidized ATP (OxATP), commonly used as $P2X_7$ antagonist. On the contrary, Supłat-Wypych et al. provided evidence

that $P2X_7$ receptors were not activated in C6 cells (Supłat-Wypych et al. 2010). In the absence of extracellular calcium, BzATP generated an increase of $[Ca^{2+}]_i$ *via* Ca^{2+} release from intracellular stores. In the presence of extracellular calcium, BzATP established a biphasic Ca^{2+} response, in a manner typical for P2Y metabotropic receptors coupled to G_q protein that involves PLC activation and the capacitative calcium entry. Moreover, OxATP blocked not only BzATP- but also UTP- induced intracellular Ca^{2+} elevation and brilliant blue G, a selective antagonist of the rat $P2X_7$ receptor, was without effect on the kinetics of calcium signals. Furthermore, neither BzATP (300 μM) nor ATP (3 mM) opened large transmembrane pores. All the above observations, together with the lack of response of UTP-desensitized cells to BzATP and *vice versa*, provide support for the activation of P2Y receptors, most probably the $P2Y_2$ receptor, by BzATP.

Taken together, *in vitro* studies suggest that in glioma C6 cells functionally active $P2X_7$ receptors do not exist. It is well known that stimulation of $P2X_7$ receptors *via* prolonged ATP exposure causes often apoptotic fate of the cell, due to massive Ca^{2+} entry. These facts, together with the shift between $P2Y_1$ and $P2Y_{12}$ receptors expression during long-term serum starvation might belong to the same self-sufficient mechanism, important for survival, up-regulated growth and invasiveness of glioma C6 cells.

On the other hand, in the *in vivo* study, Ryu et al. using immunohistochemical analysis observed colocalization of the $P2X_7$ receptor with tumor cells and microglia 2 weeks after intrastratial injection of C6 cells into rat brains (Ryu et al. 2011). Intravenous administration of the $P2X_7$ receptor antagonist, brilliant blue G, reduced tumor volume by 52 %. These results suggest that, *in vivo*, $P2X_7$ might have a different function than opening a channel and generating Ca^{2+} influx and that the $P2X_7$ receptor could be involved in signaling pathway(s) contributing to glioma growth. Moreover, brilliant blue G administrated in such a way can also influence $P2X_7$ receptors in other cells, like microglia, the activity of which is crucial to the tumor growth and invasion (Sliwa et al. 2007, see also Sect. 7.2.2).

3.8 Concluding Remarks

Rat glioma C6 is a tumoral cell line of glial origin. It has biological features of astrocytes and gliomas. One of such features is the expression of $P2Y_1$ receptors, commonly present in astrocytes and neuronal healthy cells but not in neuronal cancer cells. In C6 cells, $P2Y_1$ receptors are present and functionally active; however, their expression is highly instable. This is especially visible during serum withdrawal, when the expression of $P2Y_1$ receptors gradually decreases, and becomes undetectable after very long time of serum starvation. In contrast, $P2Y_{12}$ receptors, coupled to G_i protein and negatively linked to adenylate cyclase, are massively expressed during unfavorable conditions of serum deprivation. Both these receptors are stimulated by the same agonists. The decrease in $P2Y_1$ receptor expression is not related to the fact that these receptors induce signaling *via* G_q protein, PLC stimulation and Ca^{2+} mobilization since the expression of $P2Y_2$ receptors, acting through the same signaling cascade, is not changed during serum deprivation. The experiments with siRNA show that the shift between $P2Y_1$ and $P2Y_{12}$ expression observed during serum withdrawal is not due to a concerted cell response, but rather to the lack of serum which differentially affects $P2Y_1$ and $P2Y_{12}$ synthesis. Nevertheless, long-term serum withdrawal seems to be a convenient tool for functional studies of these receptors.

Thus, as far as receptors are concerned, the characteristic features of glioma C6 cells are: (1) instable expression of $P2Y_1$ and (2) massive expression of $P2Y_{12}$ receptors. As to the second messengers, such characteristic features are: (1) very low basal level of cAMP, (2) constitutively active PI3K/Akt signaling pathway, (3) very active ERK1/2 signaling. All these cellular traits cooperate to maintain sustained proliferation of C6 cells.

In platelets, $P2Y_1$ and $P2Y_{12}$ receptors cooperate in ADP-induced platelet aggregation. In glioma C6 cells, such cooperation is more complicated. It takes part in the stimulation of ERK1/2 activity, although the role of $P2Y_{12}$ in this process is predominating. On the other hand, the effects of these two receptors on PI3K/Akt signaling are opposite. Both in the presence and in the absence of serum, $P2Y_{12}$ activates PI3K/Akt signaling pathway, while $P2Y_1$ inhibits it.

In C6 cells cultivated in the presence of serum, the basal activity of ERK1/2 is so high that its additional activation by extracellular nucleotides cannot be observed. During serum starvation, ERK1/2 activity strongly declines, concomitantly with cell growth arrest. On the contrary, Akt activity distinctly increases, in parallel with increasing $P2Y_{12}$ and decreasing $P2Y_1$ receptor expression. Thus, it seems that under unfavorable conditions of serum withdrawal, the most important for C6 cells metabolism is to maintain a high activity of the PI3K/Akt signaling pathway, which contributes to proliferative and invasive properties of these cells. $P2Y_{12}$ acts in favor of such activity, while $P2Y_1$ acts against it and probably therefore $P2Y_{12}$ expression prevails, whereas that of $P2Y_1$ is strongly attenuated. A similar regularity takes place in the case of the $P2Y_{14}$ receptor. In C6 cells, two forms of this receptor occur: a glycosylated one, coupled to PLC, and a non-glycosylated one, negatively coupled to adenylate cyclase. Under serum withdrawal conditions, the latter form dominates.

$P2Y_{12}$ plays also an important role in maintaining a very low cAMP level. Since high cAMP level is dangerous for glioma C6 metabolism, the cells use various mechanisms to block cAMP synthesis. One of them is the inhibition of the activity of adenylate cyclase type VI, which predominates in gliomas, by increased intracellular Ca^{2+} level. Thus, in C6 cells cultivated with serum, the increase in $[Ca^{2+}]_i$ induced by activation of $P2Y_1$ receptors may down regulate adenylate cyclase in cooperation with $P2Y_{12}$ receptors, negatively coupled to this enzyme. When the cells are serum-starved and their growth is arrested, the expression of $P2Y_1$ receptors, which inhibit the PI3K/Akt signaling pathway, decreases and the expression of $P2Y_{12}$ receptors, predominates. The latter receptors stimulate ERK1/2 and PI3K/Akt activity and during serum-starvation play an important role in the maintenance of a very low level of cAMP and sustained high activity of the PI3K/Akt cascade.

Taken together, these data show the precise intracellular machinery that act in favor of maintaining glioma growth and proliferation, as well as invasiveness. In C6 cells, nucleotide receptors are involved in all of these processes and the cross-talk between $P2Y_1$ and $P2Y_{12}$ receptors seems to be an important self-regulating mechanism that helps to preserve their tumoral features.

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