2', 3'-*O*-(4-benzoylbenzoyl)-ATP-mediated calcium signaling in rat glioma C6 cells: role of the P2Y₂ nucleotide receptor

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Abstract In this study, we examined the response of glioma C6 cells to 2',3'-*O*-(4-benzoylbenzoyl)-ATP (BzATP) and showed that the BzATP-induced calcium signaling does not involve the P2X₇ receptor activity. We show here that in the absence of extracellular Ca²⁺, BzATP-generated increase in [Ca²⁺]_i via Ca²⁺ release from intracellular stores. In the presence of calcium ions, BzATP established a biphasic Ca²⁺ response, in a manner typical for P2Y receptors. Brilliant Blue G, a selective antagonist of the rat P2X₇ receptor, did not reduce any of the two components of the Ca²⁺ response elicited by BzATP. Periodate-oxidized ATP blocked not only BzATP- but also UTP-induced Ca²⁺ elevation. Moreover, BzATP did not open large transmembrane pores. What is more, a cross-desensitization between UTP and BzATP occurred, which clearly shows that in glioma C6 cells BzATP activates most likely the P2Y₂ but not the P2X₇ receptors.

Keywords BzATP · Nucleotide receptors · P2X · P2Y · P2X₇ · Glioma C6

Introduction

Extracellular ATP acts through two families of nucleotide receptors: ionotropic P2X which are ligand-gated channels, and metabotropic P2Y, members of the G-protein coupled receptor superfamily [1, 2]. To date, the P2X receptors

subtypes have been subclassified as P2X₁ to P2X₇ [3, 4]. These receptors are nonselective cation channels which upon activation by ATP, but not UTP, evoke Ca²⁺ and Na⁺ influx coupled to K⁺ efflux, according to the transmembrane gradients of these cations [1, 2]. Activation of the P2X₇ receptor requires high ATP concentration (millimolar), but since the receptor is ten to 30 times more sensitive to the ATP analog, 2',3'-*O*-(4-benzoylbenzoyl)-ATP (BzATP), the latter compound is extensively used as this receptor ligand [3, 4]. Activation of P2X₇ by high concentration of ATP for a prolonged time generates nonselective transmembrane pores permeable to large hydrophilic molecules and is responsible for massive influx of Ca²⁺ [2–4]. In several cell types, certain P2Y receptors activated by extracellular agonists (ATP, UTP, and others) can also mediate changes in intracellular Ca²⁺ levels by activation of phospholipase C (PLC), inositol phosphate formation and release of Ca²⁺ from intracellular stores such as the endoplasmic reticulum (ER) [2–4]. Thus, both the P2X and P2Y nucleotide receptors influence intracellular Ca²⁺ concentration ([Ca²⁺]_i).

Rat C6 glioma cells are nonexcitable transformed glial cells [5, 6]. This type of cell is usually characterized by a biphasic response of Ca²⁺ signaling, mediated by inositol phosphates [6, 7]. Emptying of ER calcium stores (the first phase of response) initiates a signal to the voltage-independent calcium channels and Ca²⁺ entry across the plasma membrane—the capacitative Ca²⁺ entry (the second phase of response) then occurs [6–8].

The presence of P2Y receptors in glioma C6 cells is well documented [9–12]. We have previously shown that both the ADP- or 2MeSADP-induced stimulation of P2Y₁, and the ATP- or UTP-induced stimulation of P2Y₂, initiate typical biphasic Ca²⁺ responses [8, 10, 13–15]. In contrast, the presence and activity of P2X receptors in glioma C6

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cells is weakly documented [14]. Recently, Wei et al. [16] suggested functional expression of the P2X₇ receptor in this type of cells, as activation by BzATP led to an increase in $[Ca^{2+}]_i$. On the other hand, Carrasquero et al. [17] proposed activation of not only the P2X₇ but also the P2Y₁₃ receptors by this agonist in rat cerebellar astrocytes.

To check such possibilities, in this paper, we examined BzATP as an agonist of the P2X₇ receptor by studying Ca^{2+} signaling in glioma C6 cells. We confirmed the observation of Wei et al. [16] concerning BzATP-induced rise in the intracellular Ca^{2+} level. Nevertheless, our study revealed that in rat glioma C6 cells, Ca^{2+} response to BzATP occurs via P2Y receptors, most probably by the P2Y₂-generated capacitative Ca^{2+} signaling pathway.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), newborn calf serum (NCS), and fetal bovine serum (FBS) were obtained from Gibco BRL (Invitrogen, USA). Fura-2 AM was from Invitrogen (Invitrogen, USA). Other reagents as ATP, BzATP, periodate-oxidized ATP (OxATP), UTP, 2-methylthio-ADP (2MeSADP), Brilliant Blue G (BBG), ethidium bromide (EtBr) were purchased from Sigma Chemical.

Cell cultures

Rat glioma C6 cells were cultured as described previously [15]. For calcium measurements, cells were cultivated on 22-mm glass coverslips and, for the ethidium bromide uptake assay, in 24-well plates. Mouse satellite C2C12 myoblasts were cultured as described previously [18].

Measurement of intracellular calcium

Intracellular calcium was measured under fluorescence microscope as described by Suplat et al. [15]. Briefly, 30 min before calcium measurements, C6 glioma cells on coverslips were washed once with PBS and once with the solution containing: 137 mM NaCl, 2.7 mM KCl, 1 mM Na₂HPO₄, 25 mM glucose, 20 mM HEPES (pH 7.4), 1 mM MgCl₂, 1% bovine serum albumin and 2 mM CaCl₂ (standard buffer). Only in experiments performed in the absence of external Ca^{2+} , 0.5 mM EGTA was added instead of 2 mM CaCl₂. The cells were then incubated at 37°C for 30 min in the standard buffer with 2 μ M Fura-2 AM. Data processing was carried out using the Andor IQ 1.9 (Andor Technology, USA) and Matlab software (Matworks®). All data are expressed as the Fura-2 fluorescence 340/380 nm ratio changes against time (Δ 340/380) and the stationary

calcium level was standardized to 1 arbitrary unit, AU. Each experiment was repeated at least three times and data are expressed as means. For evoking the calcium response 100 μ M ATP, 1 mM ATP, 100 μ M UTP, 300 μ M BzATP, or 10 μ M 2MeSADP were used.

Ethidium bromide uptake assay

Ethidium bromide uptake was measured under conditions described by Fumagalli et al. [19] with some modifications. Briefly, glioma C6 and C2C12 cells were treated with the Krebs–Ringer solution buffered with HEPES (KRH) either without (control) or with one of the agonists: 300 μ M BzATP, 1 mM or 3 mM ATP for 30 min. Intracellular accumulation of EtBr was then analyzed using a laser scanning cytometer iCys (LSC; CompCyt Inc., Cambridge, MA., USA). Fluorescence excitation was provided by a 488-nm laser. The red fluorescence of EtBr was measured with long pass filters transmitting at >610 nm.

Statistical analysis

Nonparametric Mann–Whitney *U* test was used to discriminate differences between calcium responses. Differences with $p < 0.001$ were considered highly significant and marked (***), those with $p < 0.01$ were marked (**), the lack of a statistically significant difference was marked with a minus sign (–).

Results

Figure 1 shows the effect of BzATP on intracellular Ca^{2+} mobilization in individual, intact glioma C6 cells compared with that induced by ATP. When the extracellular medium contained 2 mM CaCl₂, the addition of 300 μ M BzATP caused a rapid rise in $[Ca^{2+}]_i$ (Fig. 1a). As it is shown, BzATP initiated a biphasic Ca^{2+} response—the initial transient increase in $[Ca^{2+}]_i$ (the first phase of response) followed by a long, sustained response (the second phase of response). ATP (100 μ M) had a similar biphasic effect, typical for P2Y receptors (Fig. 1b). The statistical analysis (mean value $\pm$ SE from at least three separate experiments for the indicated number of cells—*n*) revealed that the initial peak of Ca^{2+} elevation evoked by 300 μ M BzATP was 2.0 ± 0.1 AU ($n = 93$) and that evoked by 100 μ M ATP was 2.47 ± 0.14 AU ($n = 34$). Since it is well known that P2X₇ receptors require much higher concentration of ATP for activation, in subsequent experiments the effect of 1 mM ATP on changes in $[Ca^{2+}]_i$ was examined. However, Fig. 1c shows that 1 mM ATP induced the same rise in $[Ca^{2+}]_i$ as 100 μ M ATP (2.46 ± 0.01 AU, $n = 303$). Moreover, Fig. 1 shows that glioma nucleotide receptors exhibited higher affinity for ATP than

Fig. 1 Calcium response of glioma C6 cells evoked by BzATP or ATP. The agonist was added in the standard buffer containing 2 mM CaCl_2 , as indicated by an arrow. Each gray trace represents the response of a single cell and the black thick line represents the mean ratio value of the indicated number of cells (n), recorded during at least three separate experiments. **a** 300 μM BzATP, $n=93$; **b** 100 μM ATP, $n=34$; **c** 1 mM ATP, $n=303$; **d** Statistical analysis of calcium responses (**a–c**), presented as mean value (in arbitrary unit—AU) $\pm$ SE, induced by different agonists: 1 mM ATP 2.46 ± 0.01 AU, 100 μM ATP 2.47 ± 0.14 AU and 300 μM BzATP 2.0 ± 0.10 AU. Differences with $p<0.01$ are marked (**), the lack of statistically significant difference is marked with a minus sign (–)

BzATP. The difference was statistically significant with $p<0.01$ (Fig. 1d).

The concentration–response curve for BzATP is presented in Fig. 2. The calculated EC_{50} value for Ca^{2+} mobilization induced by BzATP was 99.2 μM . We have previously characterized the pharmacological properties of nucleotide receptors generating Ca^{2+} mobilization in glioma C6 cells in response to various nucleotide analogs and found that EC_{50} value for Ca^{2+} mobilization induced by ATP was 4.5 μM [14]. Thus, BzATP was less efficient in stimulating $[\text{Ca}^{2+}]_i$ than ATP.

To confirm that in glioma C6 BzATP interacted with P2Y receptors, in the next set of experiments the cells were tested in Ca^{2+} -free medium (Fig. 3a). Addition of BzATP to such cells ($n=72$) produced a rise in $[\text{Ca}^{2+}]_i$ typical for metabotropic P2Y receptors (2.19 ± 0.12 AU) associated with the depletion of ER Ca^{2+} stores (the first phase of the response). Thereafter, when the transient rise in Ca^{2+} returned to the basal level, the medium was replaced for the standard buffer without agonist. The change for a buffer containing 2 mM CaCl_2 caused capacitative Ca^{2+} influx to

the cells (the second phase of the response) (1.73 ± 0.09 AU; Fig. 3a). A similar biphasic Ca^{2+} response was induced by 100 μM UTP (Fig. 3b). In the presence of EGTA, UTP caused a transient increase in $[\text{Ca}^{2+}]_i$ to 2.38 ± 0.11 AU; following the return to the baseline the addition of CaCl_2 led to an increase of $[\text{Ca}^{2+}]_i$ to 1.83 ± 0.11 AU ($n=97$). The same kinetics of the biphasic Ca^{2+} response induced by 100 μM ATP or 10 μM ADP in glioma C6 cells was shown previously [14, 15, 20]. Thus, BzATP, similar to ATP, UTP, and ADP, generates the depletion of the ER stores that gives a signal to voltage-independent Ca^{2+} channels and gives rise to Ca^{2+} influx across plasma membrane.

Next, we examined the effect of BBG, a selective antagonist of the P2X₇ receptor, on calcium response to BzATP using the same type of experiment as described above. Control conditions were applied in the absence of the antagonist (Fig. 4a). In the experiments, 1 μM BBG was added either prior to the addition of BzATP (Fig. 4b), prior to the replacement of the medium for that with CaCl_2 (Fig. 4c), or was present during all the experiment (Fig. 4d). As it is shown, in neither case did BBG reduce the intracellular Ca^{2+}

Fig. 2 Concentration-dependent effect of BzATP on calcium response in glioma C6 cells tested in the standard buffer containing 2 mM CaCl_2 . Each point represents a relative strength of calcium response compared to that induced by 300 μM BzATP (100%) at least three separate experiments. The number of cells in each agonist concentration point averaged $n=94$ –294. $\text{EC}_{50}=99.2$ μM . Values are presented as means $\pm$ SE

store depletion or the Ca^{2+} influx, indicating that BzATP-induced Ca^{2+} rise occurred according to the mechanism of the capacitative Ca^{2+} entry.

Figure 5 shows the effect of another commonly used P2X₇ receptor antagonist, periodate-oxidized ATP (OxATP), which irreversibly blocks P2X₇ receptors at concentrations of 100–300 μM [21]. The experiments were performed in the extracellular medium containing 2 mM CaCl_2 and the effect of 300 μM OxATP on BzATP- and UTP-induced Ca^{2+} rise was examined. As it is well known, extracellular UTP does not activate any of the P2X receptors [1–4]. Figure 5a shows

that OxATP distinctly blocked the effect of BzATP by 67%, as compared to the control cells treated with BzATP alone (2.17 ± 0.03 AU, $n=648$ versus 1.39 ± 0.23 AU, $n=347$). Nevertheless, the inhibitory effect of OxATP on UTP-induced Ca^{2+} mobilizations was the same and the Ca^{2+} rise was blocked by 66% (2.75 ± 0.07 AU, $n=440$ versus 1.53 ± 0.02 AU, $n=559$; Fig. 5b). In both cases, changes were statistically significant with $p < 0.001$.

To check whether P2X₇ receptors may form large transmembrane pores in C6 cells, experiments with fluorescent dye EtBr were performed (Fig. 6). Contrary to mouse satellite C2C12 myoblasts (Fig. 6a), extended exposure of glioma C6 cells to 1 mM ATP (not shown), or to 3 mM ATP (Fig. 6b) did not result in opening of membrane pores and intracellular accumulation of the dye.

To examine the involvement of the particular P2Y receptors in BzATP-generated Ca^{2+} responses experiments with sequential additions of UTP, BzATP and 2MeSADP were carried out in the absence of extracellular Ca^{2+} . 2MeSADP is a specific agonist of ADP-sensitive receptors: P2Y₁ (coupled to G_q), P2Y₁₂ (coupled to G_i) and P2Y₁₃ (coupled to G_i) [15, 22, 23]. As it is shown in Fig. 7, a second addition of 100 μM UTP, 300 μM BzATP, and 10 μM 2MeSADP added 2.5 min after the first addition of the same agonist had no further effect on the cytosolic Ca^{2+} level. UTP treated cells failed to respond to BzATP (Fig. 7a) and similarly, BzATP treated cells failed to respond to UTP (Fig. 7c), indicating the cross-desensitization between UTP and BzATP. In contrast, UTP treated cells were capable to respond to 2MeSADP (Fig. 7b). Also, 2MeSADP treated cells were capable to respond to BzATP (Fig. 7d), although the BzATP-induced rise in $[\text{Ca}^{2+}]_i$ was smaller than those observed in Figs. 3a and 4a. These data suggested that UTP and BzATP exerted their effects through the same subtype of

Fig. 3 Calcium response of glioma C6 cells evoked by 300 μM BzATP or 100 μM UTP. The agonist was added (arrow) in the initial absence of extracellular Ca^{2+} and then the medium was exchanged for the standard buffer (arrow). Each gray trace represents the response of a single cell and the black line represents the mean ratio value of the indicated number of cells (n), recorded during at least three separate experiments. **a** 300 μM BzATP, $n=72$; **b** 100 μM UTP, $n=97$

Fig. 4 The effect of 1 μ M BBG, a selective antagonist of the P2X₇ receptor, on the two phases of calcium response evoked by 300 μ M BzATP. In each case, the agonist was added in the initial absence of extracellular Ca²⁺ (arrow), then the medium was exchanged for the standard buffer containing 2 mM CaCl₂ (arrow). BBG was added either 3 min prior to the addition of BzATP (**b**); after termination of the first phase of the response, 3 min prior to the exchange of the medium (**c**); or was present during all experiment (**d**). Each gray trace represents the response of a single cell and the black line the mean ratio value of the indicated number of cells (*n*), recorded during at least three separate experiments. Mean value (in arbitrary unit—AU) $\pm$ SE for first and second phase of calcium responses averaged: **a** first phase: 1.58 ± 0.04 AU and second phase: 1.47 ± 0.06 AU, *n*=75; **b** first phase: 1.69 ± 0.11 AU and second phase: 1.45 ± 0.08 AU, *n*=70; **c** first phase: 1.52 ± 0.05 AU and second phase: 1.48 ± 0.04 AU, *n*=77 and **d** first phase: 1.68 ± 0.07 AU and second phase: 1.45 ± 0.03 AU, *n*=73. In all cases, differences between control and experimental conditions were statistically insignificant

the P2Y receptor, while 2MeSADP acts through a different one.

Discussion

P2X receptors form a class of closely related ligand-gated channels but their physiological role is difficult to study because of the lack of type-selective agonists and antagonists. One of the P2X agonists is BzATP, often used as a specific ligand of the P2X₇ receptor. However, other P2X receptors may also be activated by this compound [3, 4, 21] and there is evidence indicating that BzATP may activate P2Y receptors [17, 24, 25]. Among the crucial features that distinguish P2X₇ from other nucleotide receptors are its low affinity to

ATP ($EC_{50}=0.3\text{--}1.8$ mM) and the finding that the effect of BzATP is about ten- to 30-fold more potent than that of ATP [3, 4, 21]. Therefore, to study this receptor activity, ATP in the concentration greater than 100 μ M should be used. However the results of this study showed that in C6 cells ATP had a similar effect on $[Ca^{2+}]_i$ at concentration of 100 μ M and 1 mM, while that the potency of BzATP was lower than that of ATP. Thus, none of the above two criteria was fulfilled.

The next criterion that discriminates P2X₇ is the kinetics of calcium responses. In this paper, we compared the Ca²⁺ responses induced by BzATP (300 μ M, used as P2X₇ agonist) with those generated by ATP and UTP (100 μ M, used as P2Y₂ agonists). We have previously indicated that glioma C6 cells express nucleotide receptors of molecular and pharmacological identity consistent with that of P2Y₂.

Fig. 5 Effect of 300 μM OxATP on calcium responses of glioma C6 cells evoked by 300 μM BzATP (**a**) or 100 μM UTP (**b**) in the standard buffer. The cells were incubated with OxATP for 2 h prior to intracellular Ca^{2+} level measurements and the antagonist was present during all experiment. **a** Calcium responses evoked by 300 μM

BzATP. *Black line* control cells, $n=648$; *gray line* cells preincubated with 300 μM OxATP $n=347$. **b** Cells treated with 100 μM UTP. *Black line* control cells, $n=440$; *gray line* cells preincubated with 300 μM OxATP $n=559$. In both cases, $p<0.001$ (***)

Their activation by 100 μM ATP and 100 μM UTP led to a biphasic intracellular Ca^{2+} mobilization [14]. The present study showed that BzATP-induced Ca^{2+} elevation started with an initial peak followed by a long sustained phase in the manner typical for the P2Y metabotropic receptors. Furthermore, exposure of cells to BzATP in Ca^{2+} -free medium caused the release of Ca^{2+} from the ER stores. After subsequent replacement of the extracellular medium for the one containing calcium ions, Ca^{2+} flowed to the cells *via* mechanism dependent on the filling state of the ER stores. The biphasic process that involves PLC stimulation and inositol-1,4,5-trisphosphate (IP_3) formation causes release of Ca^{2+} from the ER store *via* IP_3 receptors (the first phase of the response) and is followed by capacitative Ca^{2+} entry *via* voltage-independent Ca^{2+} channels in the plasma membrane, as a consequence of the depletion of the ER store (the second phase of the response) [6, 7]. Such process is ubiquitous in

all nonexcitable cells and has been previously described by us in glioma C6 as the result of ATP and UTP-evoked P2Y₂ receptor activation [8, 14, 15, 20]. Similarly, we have demonstrated that in C6 cells, thapsigargin, acting without IP_3 production as a specific inhibitor of the ER Ca^{2+} -ATPase, initiated Ca^{2+} response consistent with the capacitative model of Ca^{2+} entry, in which the depletion of intracellular stores provides a signal for the transmembrane Ca^{2+} influx [8, 20]. Thus, we suggest that the initial Ca^{2+} transient generated by BzATP in glioma C6 cells is associated with the depletion of intracellular stores followed by sustained elevation of Ca^{2+} which occurs by the capacitative Ca^{2+} entrance mechanism typical for P2Y metabotropic receptors coupled to PLC.

It is worth adding that the kinetics of Ca^{2+} response observed by Wei et al. [16] in glioma C6 cells treated with 300 μM BzATP in the presence of extracellular Ca^{2+} , had the

Fig. 6 Ethidium bromide uptake in C2C12 cells (**a**) and glioma C6 (**b**) after 30 min pretreatment with 3 mM ATP. For details, see “Materials and methods” section

Fig. 7 Effect of sequential addition of 100 μ M UTP, 300 μ M BzATP and 10 μ M 2MeSADP on glioma C6 cells Ca^{2+} response measured in the buffer without CaCl_2 . Each *gray trace* represents the response of an individual cell and the *black line* the mean ratio value of the indicated number of cells (n), recorded during at least three separate experiments. Additions of agonists are indicated by *arrows*. **a** $n=105$; **b** $n=74$; **c** $n=55$; **d** $n=61$

same character as that presented in our study. However, the fact that BzATP has been able to generate Ca^{2+} response, which in addition was blocked by OxATP, was interpreted by the authors as a proof for the functional activity of P2X_7 receptors. A similar conclusion concerning functionality of the P2X_7 receptor was reached in the case of cortical astrocytes [19, 26]. On the contrary, Fisher et al. [25], using also the cultured cortical astroglia, provided evidence that functional P2X_7 receptors were not activated by this agent, since BzATP increased $[\text{Ca}^{2+}]_i$ not only in the presence but also in the absence of extracellular Ca^{2+} . The lack of P2X_7 activity upon stimulation by BzATP was also observed in rat hippocampal astrocytes [26]. All of these data indicate substantial controversy over the use of BzATP as the P2X_7 receptor agonist.

Additionally, the specific feature of the P2X_7 receptor is its ability to generate the opening of cell membrane pores that are permeable to large cationic dyes [21]. However, in our hands, neither BzATP nor ATP caused pronounced opening of membrane pores in C6 cells. Similarly, in cultured cortical astroglia Fischer et al. [25] demonstrated the lack of BzATP and ATP effect on the opening membrane pores, whereas other research groups, using either cortical astrocytes [19, 26] or C6 cells [16] presented opposite data. More recently, to explain those controversies, it was proposed that proteins other than the receptor-channel itself (e.g., pannexin-1) might be responsible for the opening membrane pores following the P2X_7 receptor activation [27].

Glioma C6 cells are often used as a model system for studying the biology of brain tumors. Braganhol et al. [28] studied P2 receptors in this cell line in culture and after implantation to rat brain and then their further growth as a primary culture (ex vivo culture model). The authors found that implantation changed the cell morphology for that characteristic of glioblastoma multiforme. However, both in the C6 glioma cell line and the C6 ex vivo culture mRNA P2X_7 was not expressed. Thus, in contrast to Wei et al. [16] the authors concluded that the P2X_7 receptor was absent in this cell line [28].

Nevertheless, even if this receptor protein was present in C6 cells, the results of our study do not allow for the assumption that P2X_7 could be activated by BzATP. Just the opposite, we postulate that BzATP activates P2Y receptors. If BzATP interacted with P2Y receptors, then OxATP used by Wei et al. [16] as a P2X_7 receptor antagonist should block the activity of P2Y receptors. Hence, we used UTP which activates P2Y_2 and P2Y_4 receptors equipotently with ATP, but in contrast to ATP does not activate any P2X receptors [2, 4]. Our results clearly showed that OxATP reduced both the BzATP- and UTP-induced Ca^{2+} mobilization. Moreover, BBG, a selective antagonist of the P2X_7 receptor affected neither the BzATP-generated Ca^{2+} release from the ER store nor the

capacitative Ca^{2+} entry. Therefore, on the basis of all these data, we suggest that in glioma C6 cells, BzATP-evoked Ca^{2+} mobilization is the result of metabotropic but no ionotropic receptors activation.

Recently, Carrasquero et al. [17] measuring calcium responses in rat cerebellar astrocytes postulated that BzATP activated both the metabotropic P2Y_{13} and the ionotropic P2X_7 receptors. P2Y_{13} activation was represented by the initial, transient phase of calcium response, while P2X_7 activation by the second, sustained phase. It is rather controversial because one of the main criteria to distinguish ionotropic receptors, and among them P2X , from metabotropic receptors is the time course of the response. The response occurs within milliseconds for ionotropic receptors activation and is much slower, occurring in minutes, for metabotropic receptors activation [3, 29]. On the other hand, Wildman et al. [24] presented evidence that BzATP together with ATP and UTP fully activated P2Y_2 receptors. On the contrary, P2Y_4 was activated by ATP and UTP, whereas BzATP was an antagonist of its activity. Receptors were expressed in *Xenopus* oocytes and their activation was recorded electrophysiologically *via* calcium dependent chloride currents.

In conclusion, the results of our study provide several lines of evidence that in glioma C6 cells BzATP activates the P2Y receptor linked to the stimulation of PLC and Ca^{2+} release. The lack of the response of UTP-desensitized cells to BzATP as well as the lack of response of BzATP-desensitized cells to UTP provides a strong support for the presence of a common receptor, most likely P2Y_2 , activated by both agonists. The role of the P2Y_{13} receptor in BzATP-induced calcium elevation remains to be clarified.

It is also well known that stimulation of the P2X_7 receptor *via* prolonged ATP exposure causes formation of megachannels and often, due to massive Ca^{2+} entry, an apoptotic fate of the cell [3, 4, 21, 30]. However, Braganhol et al. [28] presented evidence that prolonged incubation of the C6 glioma cell line or the C6 ex vivo culture with higher ATP concentration (up to 5 mM) did not affect significant decrease in cell proliferation. These data support the results of our previous studies which showed the self-sufficient mechanism for survival of C6 cells under inhospitable conditions, i.e., long-term serum-starvation [9, 31]. Thus, one could suppose that the pro-survival processes described in C6 glioma cells might switch off the Ca^{2+} signaling pathways induced by the P2X_7 receptor activation.

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