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P2X7 receptor activity landscape in rat and human glioma cell lines

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P2X7 is a commonly expressed purinergic receptor, which functions as a cation-permeable channel in the plasma membrane. In certain circumstances, the receptor may also form a large transmembrane pore what results in cell death. P2X7 receptors control numerous physiological and pathological cellular processes and their overexpression is often associated with cancer progression. As nucleotides are important signaling molecules in the central nervous system, P2X7 plays also an important but ambiguous role in glioma biology with contrary observations originating from different glioma models. Therefore, the aim of our research was to investigate P2X7 receptor expression and functions in three human (U-87 MG, U-138 MG, U-251 MG) and one rat (C6) glioma cell lines. Although the receptor mRNA and protein were present in all the studied cells, we found profound differences in their level. We also encountered a problem with one human cell lines authenticity (U-87 MG) and excluded it from most of the experiments. Interestingly, there was no clear dependency between P2X7 receptor level, calcium signal and pore formation ability in the studied glioma lines. In U-138 human cell line, the receptor seemed to be inactive, while in U-251 human and C6 rat cell line its activation resulted in calcium influx and large pore formation. However, the viability of studied cells upon the administration of specific P2X7 agonist – BzATP – was not affected for U-138 and U-251, whereas for C6 cells a stimulatory effect was observed. Our results stress the variability of P2X7 signaling in glioma models and the need for future research which would take into account the complicated landscape of the receptor signaling in the brain.

Key words: nucleotide receptors, P2X7R, glioma, human brain tumors, calcium signaling

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Abbreviations: ATP, adenosine triphosphate; BBG, brilliant Blue G; CNS, central nervous system; BzATP, 2′/3′-O-(4-benzoylbenzoyl) adenosine-5′-triphosphate; LDH, lactate dehydrogenase; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), PI, propidium iodide

INTRODUCTION

Nucleotides are an important class of signaling molecules in the nervous system, what was discovered already fifty years ago (Burnstock, 1972; Burnstock, 2007). ATP and other extracellular nucleotides released by neurons (Lopatář *et al*., 2015) as well as glia (Illes *et al*., 2019) are responsible for the communication between different CNS cells (Abbracchio *et al*., 2009): metabotropic, G-coupled P2Y receptors (Abbracchio *et al*., 2006; von Kügelgen, 2019) and P2X receptors which serve as plasma membrane ion channels (Kawate, 2017). All nucleotide receptors are characterized by different ligand affinity and downstream signaling properties. The extracellular nucleotide landscape is further complicated by the activity of ectoenzymes converting nucleotide species one to another in the extracellular space (Yegutkin, 2008). This well-established, general knowledge is however still expanding and forms a complicated network of crosstalking signaling pathways (Mutafova-Yambolieva & Durnin, 2014; Burnstock, 2016).

In this paper, we concentrate on P2X7, formerly known also as P2Z receptor (Surprenant *et al*., 1996), which is one of the ionotropic nucleotide receptors, present in most of the tissues. P2X7 acts as homotrimeric ATP-gated non-selective cation channel, leading to Ca^{2+} and Na^{+} influx and K^{+} efflux. The receptor is involved in many various processes: immune response, inflammation, cell death and proliferation, cell metabolism and autophagy among others (Young & Górecki, 2018). Interestingly, prolonged activation of this receptor in somatic cells provides to pore formation in plasma membrane permeable to large molecules (Surprenant *et al*., 1996). Uncontrolled and massive Ca^{2+} entry induces mitochondrial membrane depolarization which eventually leads to the apoptotic cell death. However, the mechanism of pore formation is not fully understood. Some reports suggest a role of another protein – pannexin-1 – in this process (Xu *et al*., 2012), while others claim that dilation of the P2X7 channel is sufficient for the pore opening and may be an effect of cell swelling (Anastacio Alves *et al*., 2014). Recent work revealed also a possible role of plasma membrane lipids in the pore formation (Karasawa *et al*., 2017).

P2X7 is also expressed in central nervous system cells: microglia, astrocytes and oligodendrocytes, whereas its presence in neurons is still a subject of the debate (Illes *et al*., 2017; Miras-Portugal *et al*., 2017; Kaczmarek-Hajek *et al*., 2018). Importantly, glia and neurons use the extracellular ATP to communicate and to maintain the homeostasis in the brain; moreover, ATP may be also released from damaged CNS cells (Rivera *et al*., 2016). It is not surprising then, that ATP-gated P2X7 is involved in many physiological and pathological processes in CNS, such as neurotransmission (Sperlágh *et al*., 2002), phagocytosis during brain development (Gu & Wiley, 2018), immunological cells infiltration (Panenka *et al*., 2001) as well as neurodegenerative diseases, psychiatric disorders and neuroinflammation (Sperlágh & Illes, 2014).

P2X7 receptor signaling is also believed to play a crucial role in gliomas which represent most of the CNS malignant tumors and are characterized with very poor prognosis (Jiang & Uhrbom, 2012). The influence of P2X7 on glioma progression is far from unambiguous. Results obtained from mice glioma GL261showed that P2X7 expression is necessary for radiation-induced cell death (Gehring *et al*., 2015). Similar results on the same glioma model were obtained by (Tamajusuku *et al*., 2010) where the researchers observed necrotic death of the cells after P2X7 stimulation. On the other hand, studies on rat glioma model C6 mostly showed the supporting role of P2X7 in the glioma progression. (Wei *et al*., 2008) observed elevated P2X7 expression after the receptor stimulation, which also resulted in calcium influx and large P2X7 pore formation. However, instead of an increase in cell apoptosis, activation of P2X7 resulted in cell proliferation and migration boost. Using the same cell line in *in vivo* experiments, (Ryu *et al*., 2011) showed an increase in P2X7 expression in microglia and astrocytes associated with the tumor. The researchers also suggested a potential therapeutic profit from inhibition of P2X7 receptors by its specific antagonist BBG (Brilliant Blue G) as the tumors exposed to the inhibitor were smaller and correlated with a significantly longer lifetime of the treated rats when compared to the control. Similarly, in human glioma samples obtained from patients, (Monif *et al*., 2014) observed P2X7 overexpression in the tumor when compared to the normal brain, especially in the glioma cells on the tumor periphery, but also in the microglia and, to a lower extent, in the astrocytes associated with the tumor. Concordantly, it is known from long time that activated microglia show increased activation of P2X7 receptor in various pathological states (Ferrari *et al*., 1999; Ferrari *et al*., 1997; Hide *et al*., 2002) and it is well established that it contributes to the glioma-supporting microenvironment (Gieryng *et al*., 2017).

In the light of somehow contrary observations coming from different glioma models, it is undoubtedly worthy to verify and further investigate the different molecular mechanisms of P2X7 signaling in the tumor progression, especially using human tissues and cells. P2X7 role in glioma therapy may be even more important in the light of (Martins *et al*., 2009) observations that various chemotherapeutic agents induce ATP release from different cancer cells. Thus, the aim of the present paper was to investigate the P2X7 expression and functioning in three human glioma cell lines and compare them to rat glioma model, C6 cell line as well as to assess the influence of P2X7 receptor stimulation on the viability of the cells.

MATERIALS AND METHODS

Cell culture. Rat glioma cell line C6 and human glioma lines (U-251 MG, U-138 MG, U-87 MG) obtained from ATCC (C6), ECACC (U-251 MG and U-87 MG) and DSMZ (U-138 MG) were cultured in DMEM High Glucose medium (4.5 g/l glucose, Thermo Fisher Scientific Inc., USA), supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific Inc., USA). Cells were grown in standard conditions (37°C, 5% CO_2, 70% humidity). Cell lines were routinely tested to detect mycoplasma contamination using PCR Mycoplasma Detection Kit (Applied Biological Materials Inc., Canada), according to manufacturer's instruction (see: Supplementary file 2 at https://ojs.ptbioch.edu.pl/index.php/abp/). All the experiments were performed 1–4 weeks after thawing the frozen cell lines. The cell lines used in this work have different population doubling time. C6 cell line divides fastest and before the experiments we performed 2 to 3 passages after thawing the cells. On the other hand, U251 and U138 divide slower and therefore we performed the experiments after at least 1 passage after thawing.

RNA isolation and reverse transcriptase PCR. Total RNA was extracted using GeneMATRIX Universal RNA Purification Kit (EURx, Poland). DNA removal from RNA samples was performed by treatment with DNase I (EURx, Poland). cDNA was synthesized using 1 µg RNA and smART First Strand cDNA Synthesis kit (EURx, Poland) according to the manufacturer's instructions. Random hexamers were used as primers for the reverse transcription reaction. Obtained cDNA served as a template for PCR reaction using primers specific for P2X receptors (Table 1). The reaction mix was as follows: 1X Buffer Gold, 2.5 mM MgCl2, 200 uM dNTP Mix, 0.5 µM forward and reverse primers, 1 U Gold Taq Polymerase (Syngen Biotech, Poland), 2 µl cDNA (10% of the total post-RT mixture), in a total volume of 20 µl. PCR reactions were performed in MJ Mini Thermal Cycler (Bio-Rad Laboratories, Inc., USA) using the following temperature profile: initial denaturation at 95°C for 5

Table 1. PCR primers used for assessment of human glioma P2X receptors expression

Receptor with Esembl genome browser ID	Sequence 5'>3'	Product length (bp)
P2X1 ENSE00003600494	F: CACATCTACACCCCCAAGCACACA R: TCATTCCCTTTATTGTACTCCACCCGT	256
P2X2 ENSE00001373821	F: GTGGTGTCATCGGGGTCATT R: TGAAGTTGTAGCCTGACGAG	129
P2X3 ENSE00003538454	F: GGAAACCTCCTTCCCAAC R: AAAATCCTGCCCCGCAAA	123
P2X4 ENSE00003585161	F: GGAATATCCTTCCCAACATCA R: CTCCACGGCCATGTCCT	140
P2X5 ENSE00002403617	F: GCCTAGAAGACAGTTCCCAGGAGG R: ACACAGATCCGTTCCCCTTCT	168
P2X6 ENSE00001347509	F: AAAGCAACCGCCAACTCTGT R: GCAAGTGGGTGTCAGAAC	163
P2X7 ENSE00001319811	F: GGACTTCACAGATTTGTCC R: TGGCTTCAGTAAGGACTCT	492

minutes, followed by 45 cycles of denaturation at 95°C for 1 minute, annealing at 55–60°C for 1 minute and elongation at 72°C for 1 min. A final elongation was carried out at 72°C for 10 min. PCR products were identified using 1.5% agarose gels containing SERVA DNA Stain G (SERVA Electrophoresis GmbH, Germany) and visualized under ultraviolet light. Gel images were recorded using G:Box Chemi HR16 BioImaging System (Syngene, UK), densitometry was performed using Fiji distribution of ImageJ (Schindelin *et al*., 2012).

Protein Extraction and Western blot analysis. Cells (1×10^6) were lysed using RIPA buffer (Thermo Fisher Scientific Inc., USA) supplemented with protease inhibitor mixture (Complete™, Roche Applied Science, Switzerland). The protein concentration of whole cells extracts was measured using the Protein Assay Kit (Bio-Rad Laboratories, Inc., USA). Samples were resolved using SDS-PAGE on 8% acrylamide gel (30 μg protein per well) and blotted onto nitrocellulose membrane using a Trans-Blot Turbo transfer system (Bio-Rad Laboratories, Inc., USA). Membranes were blocked in fat-free 5% milk in TTBS (0.25 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.1% Tween-20) and incubated overnight with primary antibody in 4°C. Following primary antibodies were used: anti-P2X7 (Alomone Labs, Israel; 1 : 250), Monoclonal Anti-β-Actin−Peroxidase (Sigma-Aldrich, Germany, 1:50 000). Anti P2X7 primary antibody was detected using anti-rabbit secondary antibodies conjugated with HRP (Merck Millipore, Germany, 1:10 000). The signal from both primary antibodies was visualized using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific Inc., USA). Immunodetected proteins were imaged on X-Ray film (Kodak), densitometry was performed using Fiji distribution (Schindelin *et al*., 2012) of ImageJ (Rueden *et al*., 2017) and integrated density normalized to actin band was averaged between the repeats.

Calcium measurements. Calcium measurements were performed as described previously (Onopiuk *et al*., 2010), using Fura-2 AM ratiometry (Grynkiewicz *et al*., 1985). Briefly, glioma cells (1.5×10^4) were seeded on rectangular glass coverslips in 35 mm dishes the day before the experiment. Cells (50–70% confluent) were loaded with 50 μM Fura-2 AM (ThermoFisher Scientific Inc., USA) in serum-depleted culture medium for 30 min at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cells were then washed twice with the solution composed of 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 25 mM HEPES, 130 mM NaCl, 1 mM pyruvate, 5 mM D-glucose, and 0.1 mM $CaCl_2$, pH 7.4 and the coverslips were mounted in a cuvette containing 1.5 ml Ca^{2+}-containing assay solution (as above but with 2 mM $CaCl_2$) and maintained at RT in a RF5001PC spectrofluorimeter (Shimadzu Corp., Japan). Fluorescence was measured at 510 nm with excitation at 340 and 380 nm every 1.4 s for at least 300 s. Excitation and emission slit width was 2.5 nm and 20.0 nm, respectively. Calcium signal was evaluated as changes in F_{340}/F_{380} fluorescence intensity ratio. Specific treatments were applied after at least 60 seconds from the start of the measurement to alleviate the effect of dye bleaching. The cells were treated with 300 μM BzATP (Jena Bioscience, Germany).

Propidium iodide dye uptake. Glioma cells were seeded on 6-well plate (5×10^5/well) and cultured for 24 hours in standard conditions. Cells were subsequently harvested by trypsinization and washed in 1x PBS. Next, cells were collected in eppendorf tubes and incubated with propidium iodide (Sigma-Aldrich, Germany) solution (1 mg/ml) for 15 minutes or co-treated with 100 μM BzATP (Jena Bioscience, Germany) and propidium iodide solution (1 mg/ml) for 15 minutes. After incubation, cells were washed in 1x PBS and lysed using distilled water to destroy the plasma membrane. Dye fluorescence (ex. 488 nm, em. 617) was measured using Infinite M1000 PRO plate reader (Tecan Trading AG, Switzerland).

MTS assay. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) metabolic activity assay was performed to measure cell viability and growth (Cory *et al*., 1991). Cells were seeded (5×10^3/well – U-138, U-251; 1×10^4/well – C6) on 96-well plate. 24 hours later cells were switched to serum-free DMEM medium supplemented with Gibco N2 supplement (Thermo Fisher Scientific Inc., USA). Cells were treated with 100 μM BzATP (Jena Bioscience, Germany), 100 nM BBG (Sigma-Aldrich, Germany) or the combination of both for 24 hours. Cell viability was evaluated with the MTS assay kit (Promega, USA) according to the manufacturer's instructions. The absorbance was read using Sunrise plate reader (Tecan Trading AG, Switzerland) at 490 nm.

LDH assay. LDH (lactate dehydrogenase) assay was performed to measure cell death in glioma cells. Cells were seeded (5×10^3/well – U-138, U-251; 1×10^4/well – C6) on 96-well plate. 24 hours later cells were switched to serum-free DMEM medium supplemented with Gibco N2 supplement (Thermo Fisher Scientific Inc., USA). Cells were treated with 100 μM BzATP (Jena Bioscience, Germany), 100 nM BBG (Sigma-Aldrich, Germany) or the combination of both for 24 hours. The level of released lactate dehydrogenase was evaluated with CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, USA) according to the manufacturer's instructions. The absorbance was read using Sunrise plate reader (Tecan Trading AG, Switzerland) at 490 nm.

Flow cytometric detection of apoptosis using Annexin V-FITC/PI labeling. Glioma cells were seeded on 6-well plate (5×10^5/well) and cultured for 24 hours in serum-free DMEM medium supplemented with Gibco N2 supplement (Thermo Fisher Scientific Inc., USA). After that cells were treated with 100 μM BzATP. As positive control to confirmation of properly working kit we use Jurkat cells treated by UV-C for 30 seconds. After incubation cells were subsequently harvested by trypsinization and washed in 1× PBS. Next, cells were collected in eppendorf tubes and incubated with FITC conjugated Annexin V and propidium iodide (Sigma-Aldrich, Germany) according to the manufacturer's instructions. After incubation, cells were washed in 1× PBS and analyzed by Guava® easyCyte Flow Cytometer.

Statistical analysis. Plots were generated using Matplotlib 3.1.0 Python library (Hunter, 2007). Data in the box plots are presented as fold change compared to control. The boxes represent 25 and 75 percentile, solid horizontal lines represent median values and whiskers refer to ±1.5 IQR (Interquartile Range). Asterisks represent statistically significant differences in comparison to the control. *n* refers to the number of independent biological repetitions which contain at least three technical repetitions. Statistical analysis was performed on the raw data using SigmaPlot 12.3 (Systat Software Inc., USA). To assess the differences between the treatments and the control paired t-Student test was used for PI uptake assay data and Repeated-Measures One Way ANOVA followed by Holm-Sidak *post-hoc* test was used for LDH, MTS assay and Annexin V-PI staining results. Data normality and variance equality were assessed with Shapiro-Wilk test and Levene's test, respectively. Differences were considered as statistically significant at $p<0.05$.

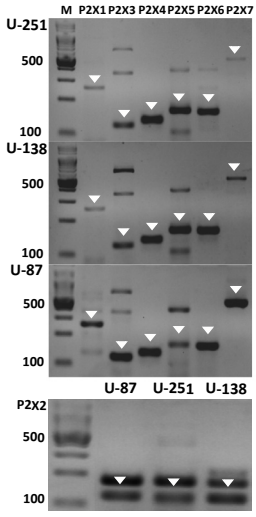

Figure 1. Expression of P2X receptors in U-251, U-87 and U-138 human glioma cell lines studied using reverse transcriptase PCR (representative result, n=3).
The bands corresponding to the predicted P2X receptors amplicon sizes are marked by arrowheads. First lane from the left corresponds to the molecular mass DNA marker; 500 and 100 refer to the length in nucleotides.

RESULTS AND DISCUSSION

Detection of P2Rs mRNA and P2X7 protein

In order to study the P2X7 receptor functioning in the human glioma cell lines, we first verified the expression of the receptor mRNA. The reverse transcription PCR assay was performed for all P2X receptors in the three human glioma cell lines (U-138, U-87 and U-251) (Fig. 1). We detected all known P2X receptors in the studied cells. In order to confirm the presence of P2X7 protein in the studied cells, we checked the protein level of P2X7 using Western Blot in the human glioma cell lines and C6 rat glioma cell line as a positive control. All the studied cell lines displayed P2X7 protein presence. However, the obtained results varied greatly between the cell lines as coarse densitometry of P2X7 bands showed differences greater than an order of magnitude (Fig. 2a). The amount of receptor protein in rat glioma C6 line was about seven times greater than the average amount from all human cell lines and almost fifty times greater than in case of U-251. We also observed a profound difference between the human gliomas with U-87 line containing sixteen times more P2X7 protein than U-251 line, while U-138 line had two and half times more of the protein than U-251 (Fig. 2b). Surprisingly, reports on P2X7 expression in human glioma cell lines are rather sparse. In line with our findings, data from The Human Protein Atlas (Thul *et al.*, 2017) confirmed very low levels of P2X7 mRNA in all three human cell lines studied by our team. Moreover, similar results were obtained by (Gehring *et al.*, 2012) on U-138 and U-251 cell mRNA. At the protein level, P2X7 presence in U-87 and U-251 cells was detected by (Ji *et al.*, 2018) using immunofluorescence. The significant difference in band intensity of P2X7 receptor in U-87 cell line in comparison to the other human cells, revealed in our study, may be a result of the problem with U-87 line authenticity reported by (Allen *et al.*, 2016). The authors claimed that the U-87 cell line publicly available in the repositories is not the same cell line that was originally isolated and, therefore, its origin is unknown – with a high probability of being

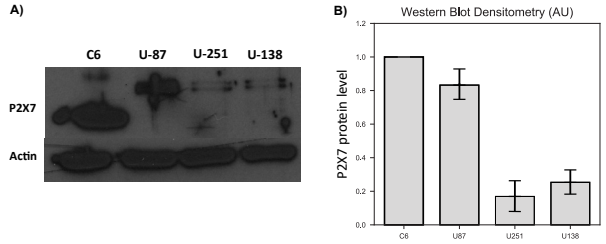

Figure 2. P2X7 receptor protein level shown on the Western Blot (representative result, n = 4).
(**A**) Representative Western Blot result, from the left: C6 rat glioma cell line U-87 human cell line, U-251 human glioma cell line and U-138 human glioma cell line. (**B**) The graph shows the results of the quantitative analysis of the Western blots, which was performed using the ImageJ program.

the central nervous system. Thus, we performed mtDNA sequencing and compared the obtained sequence to the original U-87 cells. It occurred that the cells studied by us were also the non-original ones (see: Supplementary file 1 at https://ojs.ptbioch.edu.pl/index.php/abp). In the light of the unknown origin of the cells and the vast difference in P2X7 receptor protein level when compared to our other human cell lines, we decided to omit this cell line during the further experiments.

Calcium influx and large pore formation upon P2X7 stimulation

To check the physiological activity of P2X7 receptors in the human cell lines and correlate it with receptor gene expression and protein production, we performed a fluorimetric study of intracellular calcium level using Fura-2 AM ratiometric calcium indicator with C6 cell line as a positive control, as described in Methods. The receptors were activated using BzATP, non-hydrolyzing, specific P2X7 receptor agonist. Strong differences were observed between all the studied glioma cell lines (Fig. 3). While C6 cells responded every time BzATP was administered and the strength of the response was similar, the U-251 human cell line responded erratically and U-138 presented absolutely no calcium signal upon BzATP stimulation. While P2X7-dependent calcium increase is not surprising in case of C6 cells (Wei *et al.*, 2008), to our knowledge there are no reports specifically describing presence or absence of P2X7-initiated calcium influx in the two human glioma cell lines. However, P2X7-mediated calcium signals were observed in human astrocytes, murine neural progenitor cells and rat oligodendrocyte precursor cells and all these cell types are top candidates to be the putative cells of origin for glioma (Alloisio *et al.*, 2006; Narcisse *et al.*, 2005; Jiang & Uhrbom, 2012; Wang *et al.*, 2009; Leeson *et al.*, 2018). The detailed nature of the observed signal is however not exactly clear since there are strong proofs for its metabotropic character (Suplat-Wypych *et al.*, 2010) as well as suggestions of active ATP release through P2X7 receptors (Brandao-Burch *et al.*, 2012).

Our next step in the characterization of P2X7 in human glioma cell lines was to verify the canonical large pore formation upon prolonged P2X7 activation. We performed the propidium iodide uptake assay after 24-hour receptor activation with BzATP (Surprenant *et al.*, 1996). The results of the PI uptake experiments correlated with calcium signal studies (Fig. 4), showing lack of significant difference in propidium iodide uptake after BzATP treatment in case of U-138 cells and significant

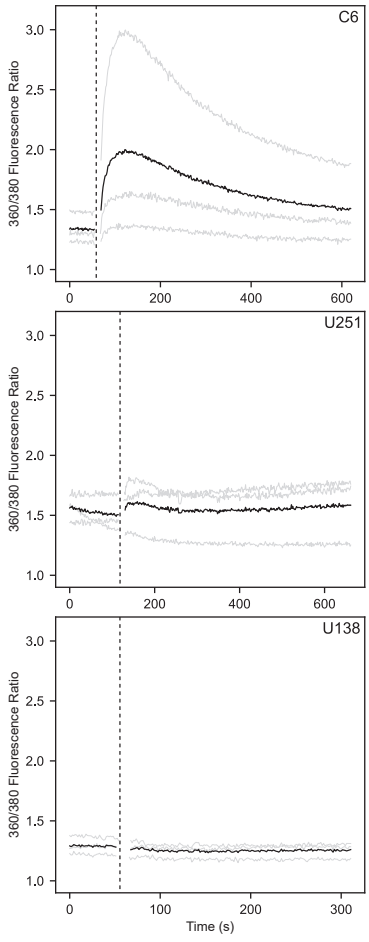

Figure 3. Calcium transient evoked by BzATP administration (dashed line) in:
C6 rat glioma cell line (**upper panel**), U-251 human glioma cell line (**middle panel**) and U-138 human glioma cell line (**lower panel**); for each cell line n=3. Gray lines represent the signals from the biological replicates, black lines represent the signal averaged among the individual replicates.

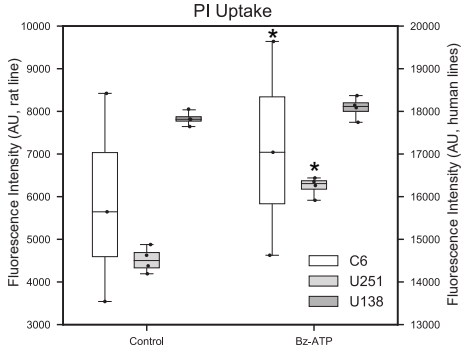

Figure 4. Measurement of propidium iodide uptake level in C6, U-251, U-138 cells.
C6 and U-251 cells showed statistically significant difference between control and BzATP-induced cells (asterisks, n=3 and n=4, respectively), suggesting the formation of large pore. U-138 control cells did not differ significantly from BzATP-treated ones (n=4). The boxes represent 25 and 75 percentile, solid horizontal lines represent median values and whiskers refer to ±1.5 IQR (Interquartile Range). Asterisks represent statistically significant differences in comparison to the control. *n* refers to the number of independent biological repetitions which contain at least three technical repetitions. The significance of the differences was determined with paired *t*-Student test: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ *vs*. the respective control.

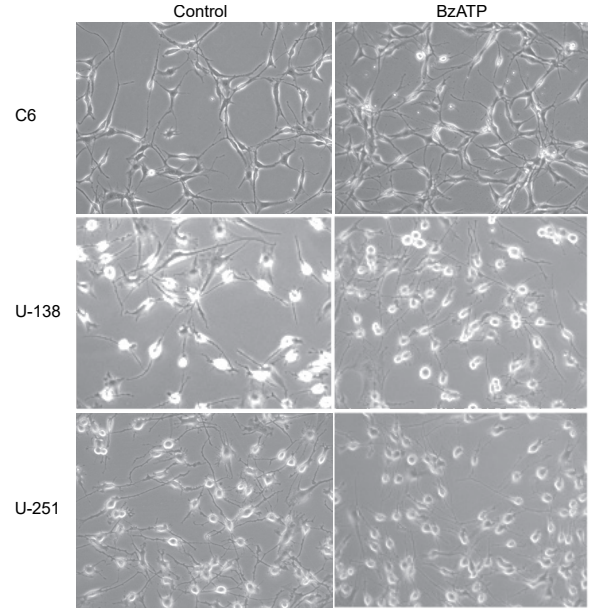

Figure 5. C6, U-251, U-138 glioma cells morphology after 24 hours of P2X7 stimulation.

uptake for C6 and U-251 cells (Fig. 5). Our observations proved that U-138 cells – on the contrary to U-251 – are not only unable to develop P2X7-mediated calcium signal, but also unable to form large membrane pore upon prolonged BzATP stimulation. This phenomenon may be explained by the presence of various P2X7 receptor isoforms in the studied cell lines. There are currently twelve known splice variants of P2X7 receptor (Cheewatrakoolpong *et al.*, 2005; Feng *et al.*, 2006). Cells expressing full-length P2X7A receptor isoform demonstrate canonical calcium signaling with plasma membrane permeabilization and cell death. In contrast, P2X7 receptor B isoform still functions as the ATP-mediated calcium channel but is unable to form the pore. There are also several reports showing the influence of the type of P2X7 receptor isoform expression pattern on cancer cells phenotype(Adinolfi *et al.*, 2010; Giuliani *et al.*, 2014). The situation is even more complicated by the possible interactions between the different isoforms expressed in the same cells, observed by (Feng *et al.*, 2006). The researchers revealed that in cervical cancer cells, endogenous, truncated P2X7j variant blocked the activation of the full-length receptor through the mechanism of heterooligomerization.

Effect of P2X7 stimulation on glioma cell lines viability

To confirm or exclude the formation of the "death pore" in the studied cell lines, we performed LDH activity assay and Annexin V/PI labeling to verify the effect of P2X7 receptor activation on cell viability. Surprisingly, BzATP stimulation did not have a negative influence on the morphology (Fig. 5) and viability (Fig. 6, Table 2, Supplementary file 3 at https://ojs.ptbioch.edu.pl/index.php/abp/) of the studied cells. Moreover, we performed proliferation assay using MTS test (Cory *et al.*, 1991). In human cell lines, P2X7 stimulation had no effect on their proliferation, whereas in rat glioma C6 cells it even led to a 29% increase in the number of viable cells (Fig. 7). (Gehring *et al.*, 2012) also observed the lack of apoptosis of U-251 and U-138 after BzATP stimulation, whereas (Ji *et al.*, 2018) obtained increased proliferation in U-87 and U-251 upon P2X7 activation. However, in the lat-

Table 2. Annexin V/ propidium iodide labeling of C6, U-251, U-138 glioma cells after stimulation with 100 μM BzATP for 24 hours.

Cell line	Treatment	Intact cells	Early apoptotic cells	Apoptotic cells	Necrotic cells
Jurkat	non treated	90.26±0.06	3.01±0.07	6.69±0.03	0.04±0.05
	UV-C, 30 seconds	4.05±0.16	18.24±0.2	77.33±0.12	0.38±0.08
C6	non treated	99.87±0.05	0.07±0.09	0.0±0.0	0.03±0.06
	BzATP, 100 μM	98.8±1.08	0.83±0.06	0.33±0.04	0.07±0.09
U-251	non treated	98.4±0.45	0.9±0.22	0. 6±0.28	0.13±0.05
	BzATP, 100 μM	97.7±1.43	1.03±0.69	0.97±0.65	0.27±0.09
U-138	non treated	98.13±0.65	1.03±0.25	0. 7±0.37	0.13±0.05
	BzATP, 100 μM	98.13±0.79	0.9±0.29	0. 77±0.42	0.17±0.12

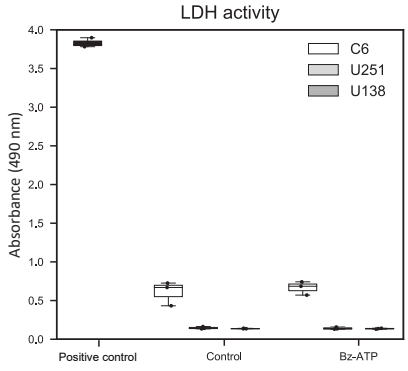

Figure 6. Viability of C6, U-251, U-138 glioma cells after 24 hours of P2X7 stimulation measured by LDH release test (n=4).
The boxes represent 25 and 75 percentile, solid horizontal lines represent median values and whiskers refer to ±1.5 IQR (Interquartile Range). Asterisks represent statistically significant differences in comparison to the control. *n* refers to the number of independent biological repetitions which contain at least three technical repetitions. The significance of the differences was determined with a one-way ANOVA with Bonferroni's post hoc test: **P*≤0.05, ***P*≤0.01, ****P*≤0.001 *vs*. the respective control.

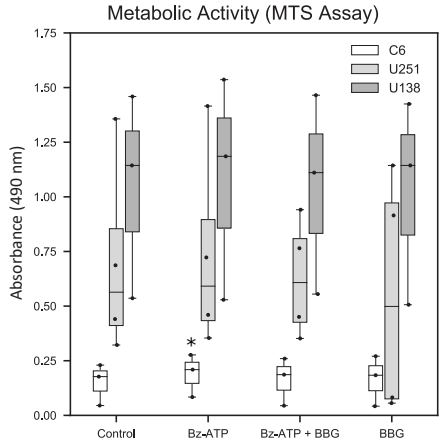

Figure 7. Proliferation of C6 (n=3), U-251 (n=4), U-138 (n=3) glioma cells upon P2X7 stimulation or inhibition was evaluated by MTS assay.
The boxes represent 25 and 75 percentile, solid horizontal lines represent median values and whiskers refer to ±1.5 IQR (Interquartile Range). Asterisks represent statistically significant differences in comparison to the control. *n* refers to the number of independent biological repetitions which contain at least three technical repetitions. The significance of the differences was determined with a one-way ANOVA with Bonferroni's post hoc test: **P*≤0.05, ***P*≤0.01, ****P*≤0.001 *vs*. the respective control.

ter case, the proliferation boost might be explained by the use of serum-supplemented cell culture in contrast to serum-free conditions applied in our experiments. While the positive influence of P2X7 on proliferation is well-known in different cells, it usually is believed to be the effect of truncated receptor isoform expression instead of the full protein and, consequently, of the inability to form the large pore (Cheewatrakoolpong *et al*., 2005). One example is from (Adinolfi *et al*., 2010) group, where activation of P2X7B in transfected HEK293 cells, naturally lacking P2X7 receptor expression, increased proliferation via activation of transcription factor NFATc1. Similar results were obtained by (Giuliani *et al*., 2014) for osteosarcoma cells. However, this explanation does not sufficiently match our observations. Interestingly, the research of (Monif *et al*., 2009) resulted in similar conclusions to ours. Using P2X7RG345Y mutant which is unable to form the large pore, the team found that P2X7 activation had a significant stimulatory effect on rat microglia activation and proliferation with the phenomenon specifically dependent on the large pore formation. The specific signaling cascades activated after the pore opening and responsible for these observations are still to be revealed though. Therefore, we may speculate that a similar mechanism could be present in glioma cell lines. Finally, P2X7 calcium and pore-forming functions might be severely altered by single nucleotide sequence differences between human individuals (Sluyter & Stokes, 2011). This may be particularly important when interpreting results from single patient-originated cell lines.

CONCLUSIONS

Our results suggest that despite the presence of P2X7 receptor mRNA and protein in all studied cell lines, its activity and physiological effects profoundly differ. In U-138 human cell line, the receptor seemed to be inactive, while in U-251 human and C6 rat cell line P2X7 activation resulted in calcium influx and, finally, large pore formation. However, the proliferation rate of U-138 and U-251 was not affected, whereas for C6 cells a stimulatory effect was observed. These results not only fit into a complicated landscape of P2X7 influence on cancer cells biology but also justify the use of human cell lines in glioma research. Moreover, future studies should take into consideration the existence of different receptor isoforms and be complemented by the animal *in vivo* experiments which can better mirror the tumor microenvironment and the complicated network of P2X7 and ATP signaling in the brain.

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