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Characterization of ATP-Induced Cell Death in the GL261 Mouse Glioma

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ABSTRACT

Gliomas have one of the worst prognosis among cancers. Their resistance to cell death induced by endogenous neurotoxic agents, such as extracellular ATP, seems to play an important role in their pathobiology since alterations in the degradation rate of extracellular ATP drastically affects glioma growth in rats. In the present work we characterized the mechanisms of cell death induced by extracellular ATP in a murine glioma cell line, GL261. ATP and BzATP, a P2X7 agonist, induced cell death at concentrations that are described to activate the P2X7 receptor in mouse. oATP, an antagonist of P2X7, blocked the ATP-induced cell death. Agonists of purinergic receptors expressed in GL261 such as adenosine, ADP, UTP did not cause any cell death, even at mM concentrations. A sub-population of cells more sensitive to ATP expressed more P2X7 when compared to a less sensitive subpopulation. Accordingly, RNA interference of the P2X7 receptor drastically reduced ATP-induced cell death, suggesting that this receptor is necessary for this effect. The mechanism of ATP-induced cell death is predominantly necrotic, since cells presented shrinkage accompanied by membrane permeabilization, but not apoptotic, since no phosphatidylserine externalization or caspase activity was observed. These data show the importance of P2X7 in ATP-induced cell death and shed light on the importance of ATP-induced cell death in glioma development. J. Cell. Biochem. 109: 983–991, 2010.

KEY WORDS: GLIOBLASTOMA MULTIFORME; GLIOMA; NECROSIS; PURINOCEPTOR; P2X7

Extracellular ATP has well described roles in several physiological processes like vascular tonus, pain sensation, neurotransmission, cell proliferation, differentiation, and death [Burnstock, 2006]. The specific purinergic receptors that mediate these effects are classified into P1 receptors (A_1, A_{2A}, A_{2B}, and A_3), which are activated by nucleosides and P2 receptors that respond to nucleotides, which are further subdivided in metabotropic P2Y (P2Y 1, 2, 4, 6, 11–14) and ionotropic P2X subtypes (P2X1-7) [Burnstock, 2006].

Among the P2X receptors, the P2X7 subtype possesses unique biological properties such as the opening of a pore through which molecules up to 900 Da can pass. However, this pore opening requires prolonged exposure to ATP in the low millimolar range and the presence of associated proteins [Surprenant et al., 1996; Pelegrin and Surprenant, 2006]. P2X7 is responsible for ATP-induced cell death in various cell types through mechanisms that involve necrotic features such as swelling, loss of membrane integrity and apoptotic features, such as shrinking and phosphatidylserine (PS) externalization [Tsukimoto et al., 2005; Taylor et al., 2008].

In the central nervous system, ATP is highly toxic to neurons in vitro and ex vivo [Morrone et al., 2005] and extracellular ATP plays an important role in neuronal death in pathological conditions such

Abbreviations used: BzATP; benzoil-benzoil adenosine 5′-triphosphate; MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; oATP; oxidized adenosine 5′-triphosphate; TNP-ATP; 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate.

Additional Supporting Information may be found in the online version of this article.

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as ischemia and trauma [Wang et al., 2004; Cavaliere et al., 2007]. Neuronal death induced by oxygen and glucose deprivation of hippocampal organotypic cultures was blocked by the P2X7 antagonist oATP [Cavaliere et al., 2004] and a broad P2X antagonist, TNP-ATP [Cavaliere et al., 2007], but knockout of the P2X7 receptor did not affect the sensitivity of mice to ischemia in vivo [Le Feuvre et al., 2003] indicating that multiple receptors may be involved in neuronal death in this pathological condition. Glial cells, on the other hand, are mostly resistant to ATP-induced cell death and ATP acts as a mitogen in astrocytes, activating pro-survival pathways such as PI3K/Akt and ERK [Lenz et al., 2000, 2001; Jacques-Silva et al., 2004]. It is interesting to notice that mouse neural progenitor cells (NPCs), which are capable of differentiation into neurons, astrocytes and oligodendrocytes, are sensitive to extracellular ATP with a pharmacological and molecular profile that suggests the involvement of P2X7. Notably, NPCs from P2X7 KO mice were much more resistant to extracellular ATP [Delarasse et al., 2009].

Gliomas are the most aggressive tumors of CNS, in part, due to their unique ability to rapidly invade the neighboring tissue, thus making surgical resection and radiotherapy much more difficult. Part of this tumor invasion seems to be due to the liberation of neurotoxic molecules, such as ATP and/or glutamate, by the gliomas and by the surrounding dying cells that open up space for tumor invasion [Takano et al., 2001]. Most gliomas examined have a rate of extracellular ATP degradation that is around three orders of magnitude lower when compared to astrocytes [Wink et al., 2003], thus allowing a build up of this molecule around the tumor. Besides the neurotoxic effect, ATP can also induce proliferation of several kinds of glioma cells lines [Morrone et al., 2003]. The importance of extracellular ATP for glioma pathobiology was confirmed by the reduction in glioma tumor size by the injection of apyrase, which degrades extracellular ATP to AMP [Morrone et al., 2006], and the striking increase in tumor size by over-expression of an ecto-enzyme that degrades ATP to ADP [Braganhol et al., 2009], suggesting that ATP is liberated and that the effect on the tumor growth depends on the nucleotide produced by its degradation.

Here we show that the mouse glioma cell line GL261 is sensitive to high concentrations of ATP and by pharmacological and genetic strategies show that this effect is mediated by the P2X7 receptor. The mechanism of cell death is mainly necrotic, not presenting clear apoptotic features. This characterization will contribute to the understanding of the importance of the purinergic system and of cell death induced by endogenously liberated molecules in tumor biology of gliomas.

MATERIALS AND METHODS

REAGENTS AND CELL CULTURE

Reagents, ATP, ADP, AMP, Bz-ATP, oATP, TNP-ATP, PPADS, suramin, reactive blue and propidium iodide (PI) were purchased from Sigma (St. Louis, MO), except when specified. Mouse GL261 glioma cell line was a generous gift of Dr. Ilker Eyüpoglu, University of Zurich. All cells were maintained in culture flask in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). In the experiments with purinergic antagonist, the drugs were added to culture medium 15 min before the nucleotide exposure, except oATP that was added 30 min before.

STABLE RNA INTERFERENCE

Mouse P2RX7 (GeneID: 18439) was knocked down (KD) by transduction of cells with lentivirus produced with the plasmid clone ID NM_011027.1-1368s1c1 from the Mission RNAi library from Sigma–Aldrich. Non-target (SHC002V) sequence was used as a control. Lentiviruses were produced by co-transfecting the Mission RNAi plasmid with the helper plasmids pRSVREV, pVSV-G and pMDLgRRE [Dull et al., 1998] in sub-confluents Hek293T cells with Superfect Reagent (Qiagen), according to the manufacture's protocol. Three days after transfection, supernatant was collected every 2 days during 1 week, filtered through a 0.45 μm membrane and used immediately or stored at −80°C. One milliliter of virus containing medium was added to target cells, also at sub-confluent stage (15,000 cells/well) in 24-well plates, together with 8 μg/ml of polybrene overnight. Cells were allowed 48 h to express the selection marker and were then selected with 5.5 μM puromycin for at least 10 days. KD was confirmed by Western blotting.

ASSESSMENT OF GLIOMA CELL VIABILITY

Loss of membrane integrity was measured through lactate dehydrogenase (LDH) release, in which the cell medium was withdrawn and transferred to an eppendorf tube on ice, centrifuged for 1 min at 10,000 rpm and an aliquot of the supernatant was used for enzymatic assay using a LDH kit available from Doles Reagentes following the manufacture's protocol. Results were expressed as percentage of 0.5% Triton X-100-induced LDH release. For PI incorporation, cells were exposed to agonists concomitantly with 1 μM PI. After the indicated times, pictures were taken on Carl Zeiss Fluorescence Inverted Microscope fitted with a standard rhodamine filter. Positive control cells received 0.5% Triton X-100. Alternatively, the number of permeabilized cells was quantified using ViaCount® Reagent, which contains PI as an indicator of permeabilization, and analyzed in a Guava EasyCyte flow cytometer (Guava Technologies, Hayward, CA).

Mitochondrial viability was assessed using the MTT assay. Briefly, glioma cells were seeded on 96 multiwell plates and allowed to grow until confluence, treated and incubated for 4 h at 37°C in MTT solution (0.25 mg/ml of MTT dissolved in Ca^{2+} and Mg^{2+}-free buffer). Formazan crystals, formed by tretrazolium cleavage, were dissolved with 100 μl of DMSO for 10 min and quantified at 560 and 630 nm using a Spectramax Spectrofotometer.

Phosphatidylserine (PS) externalization was determined by annexin fluorescent signal of Annexin-V FITC conjugate (Gibco/Invitrogen) according to the manufacture's protocol. GL261 cultures treated or not with ATP, were tripsinized, centrifuged for 5 min at 2,000 rpm and the supernatant discarded. The pellet was ressuspendend with 100 μl of Annexin Binding Buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and incubated with annexin 0.5 μl/sample and PI 18 μM for 15 min at room temperature in the dark and analyzed on Guava EasyCyte flow cytometer. Cysplatin 16.6 μM was used as positive control for apoptosis.

TABLE I. Primers for Purinergic Receptors Used at PCR (RT-PCR and qRT-PCR) Experiments

Primer	Sense	Antisense
P2X1	5′-AAGGTCAACAGGCGCAACC-3′	5′-AACACCTTGAAGAGGTGACG-3′
P2X2	5′-GTGCAGAAAAGCTACCAGG-3′	5′-GGATGGTGAAATTTGGGGC-3′
P2X3	5′-GCTGCGTGAACTACAGCTC-3′	5′-ACTGGTCCCAGGCCTTGTC-3′
P2X4	5′-CTTGGATTCCGGATCTGGG-3′	5′-GGAATATGGGGCAGAAGGG-3′
P2X5	5′-GCACCTGTGAGATCTTTGC-3′	5′-TCGGAAGATGGGGCAGTAG-3′
P2X6	5′-CAGGACCTGTGAGATCTGG-3′	5′-TCCTGCAGCTGGAAGGAGT-3′
P2X7	5′-TCCCTTTGCAGGGGAACTC-3′	5′-GTACGGTGAAGTTTTCGGC-3′
P2Y1	5′-TGTTCAATTTGGCTCTGGC-3′	5′-AGATGAAATAACTTCGCAGG-3′
P2Y2	5′-CTTCGCCCTCTGCTTCCTG-3′	5′-TTGGCATCTCGGGCAAAGC-3′
P2Y4	5′-GGCATTGTCAGACACCTTG-3′	5′-AAGACAGTCAGCACCACAG-3′
P2Y6	5′-CGCTTCCTCTTCTATGCCA-3′	5′-AGGCTGTCTTGGTGATGTG-3′
P2Y11	5′-CTTCCTCTTCACCTGCAAC-3′	5′-AGGCTATACGCTCTGTAGG-3′
P2Y12	5′-GACTACAAGATCACCCAGG-3′	5′-CCTCCTGTTGGTGAGAATC-3′
P2Y13	5′-GCCGACTTGATAATGACAC-3′	5′-ATGATCTTGAGGAATCTGTC-3′
P2Y14	5′-TCTTTTACGTGCCCAGCTC-3′	5′-CTGTCAAAGCTGATGAGCC-3′

RT-PCR ANALYSIS

Total RNA from glioma cell cultures were isolated with Trizol LS reagent (Life Technologies) and chloroform-isopropanol extraction. The cDNA was synthesized with M-MLV Reverse Transcriptase enzyme (Promega) from total RNA with oligo (dT) primer. One microliter of the RT reaction mix was used as a template for PCR using a concentration of 0.5 μM of each primer indicated below, in Table I, and 0.5 units of Taq DNA polymerase (CenBiot-UFRGS). As a control for cDNA synthesis, β-actin PCR was performed. The PCR cycling conditions were as follows: 1 min at 95°C, 1 min at 94°C, 30 s at 60°C, 45 s at 72°C and a final 10 min extension step at 72°C for 40 cycles. Products of the PCR reaction were analyzed on a 1% agarose gel stained with 1× SYBR Green for gel staining (Molecular Probes). SYBR Green I-based real-time PCR was carried out on Applied-Biosystem 7500 real-time cycler as described [Andrade et al., 2008]. All results were analyzed by the $2^{-\Delta\Delta CT}$ method [Livak and Schmittgen, 2001]. β-Actin was used as the internal control gene for all relative expression calculations. All samples were run in triplicate and the data were presented as ratio of P2X7/β-actin. The primers used for real time PCR were the same used in RT-PCR analysis.

WESTERN BLOT ANALYSIS

To determine P2X7 immunocontent, glioma cell cultures were washed twice with cold PBS and homogenized in lysis buffer (4% sodium dodecylsulfate (SDS), 2.1 mM EDTA and 50 mM Tris). Aliquots were taken for protein determination [Peterson, 1983] and β-mercaptoethanol was added to a final concentration of 5%. Forty micrograms of protein were separated on 8% SDS–polyacrylamide gel electrophoresis (Bio-Rad, CA) and electrotransferred to PVDF membranes. Membranes were blocked and further incubated with polyclonal anti-P2X7 antibody (Santa Cruz Biotechnology Inc.) 1:200, 1 h at room temperature. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:2,000) for 2 h at room temperature and the chemioluminescence was detected using X-ray films (Kodak X-Omat, Rochester, NY). The films were scanned and the percentage of band intensity was analyzed using Image J software (NIH).

STATISTICAL ANALYSIS

The data obtained was expressed as % of control and presented as mean ± SEM of at least three independent experiments unless otherwise stated. Statistical analysis was performed by Student's *t*-test and comparison among multiple groups was performed by one-way ANOVA followed by Tukey post-hoc test. Values were considered significant at $P < 0.05$.

RESULTS

MOUSE GL261 GLIOMAS ARE SENSITIVE TO ATP

ATP treatment of the mouse glioma cell line GL261 lead to cell roundup, membrane permeabilization to propidium iodine (PI) (Fig. 1A) and release of LDH, which peaked at around 2 h to reach around 70% of maximum LDH release (Fig. 1B). This lead to loss of cell viability, as measured by MTT assay, confirming that concentrations above 1 mM of ATP effectively killed these cells (Fig. 1C,D).

EXPRESSION OF PURINERGIC RECEPTOR AND PHARMACOLOGICAL PROFILE OF ATP-INDUCED CELL DEATH

GL261 gliomas express mRNA for several purinergic receptors, which include A2a, P2X1, P2X3-7, P2Y2, P2Y6, P2Y12, and P2Y14 (Fig. 2A). Activation of the A2a receptor with adenosine, P2Y2 and P2Y6, with UTP and P2Y12 with ADP, did not exert toxic effect on GL261 cells, even at the 5 mM dose (Fig. 2B). BzATP, an agonist of the P2X7 receptor, with an EC_{50} of 936 μM for the mouse receptor [Young et al., 2007], induced cell death with an EC_{50} of about 1.0 mM (Fig. 1C). TNP-ATP, considered a broad P2X antagonist, but that does not block P2X7, had no effect on ATP-induced cell death, whereas oxidized ATP (oATP), a P2X7 antagonist, completely prevented the effect of ATP in a dose-dependent fashion (Fig. 2D and Supplementary Fig. 1A), indicating that the activation of P2X7 is essential for the toxic effect of ATP in GL261. Among the non-specific purinergic antagonists tested, only PPADS partially reverted the ATP-induced death. Mg^{2+} ions, which block the effect of ATP by decreasing its binding to the receptor, also blocked the toxic effects of ATP (Supplementary Fig. 1B).

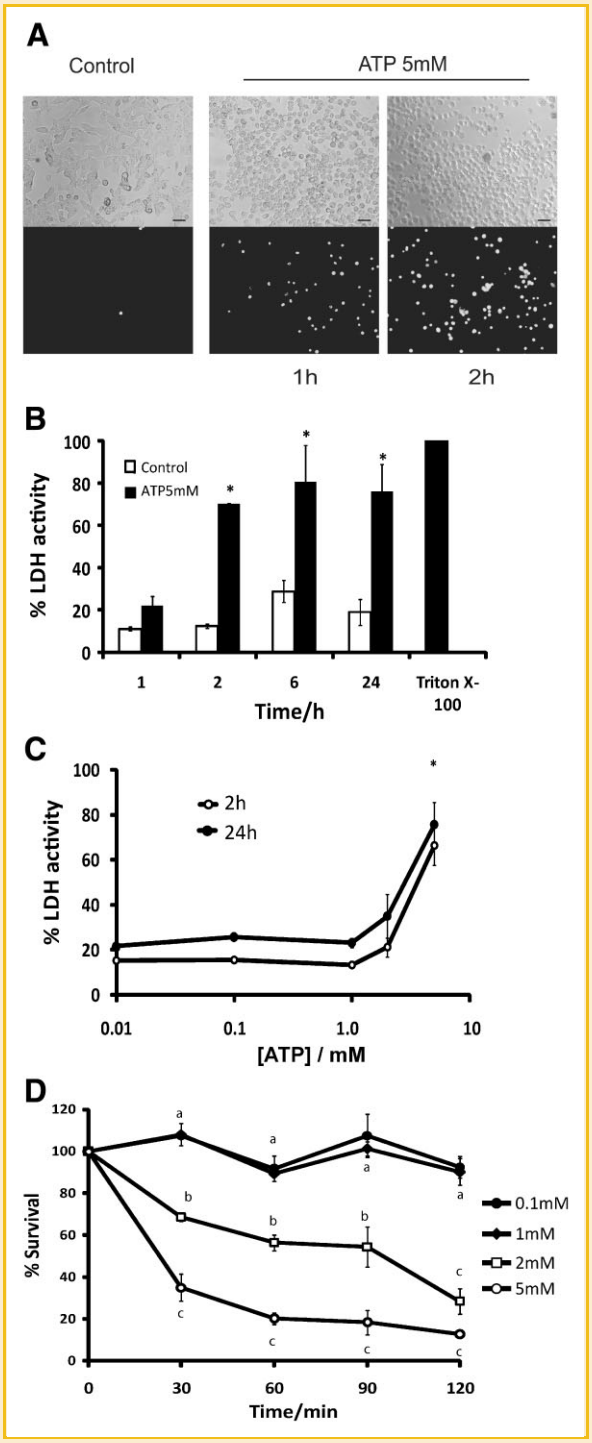

Fig. 1. The mouse glioma cell line GL261 is killed by ATP. ATP leads to cell roundup and membrane permeabilization to PI (A) and loss of membrane integrity in a dose- and time-dependent way, as measured by LDH release (B,C). The results are expressed as % of the positive control treated with 0.5% Triton X-100 ($n = 3$). $^{*}P < 0.05$; Cell viability was also accessed by MTT assay (D). a versus b versus c $P < 0.05$, a versus c $P < 0.001$. Bars represent 200 μm.

CELLS MORE SENSITIVE TO ATP EXPRESS MORE P2X7 RECEPTOR

In order to establish whether there is a correlation between sensitivity to cell death and P2X7 expression, we acutely treated GL261 cells with 5 mM ATP and collected the detached cells after 20 min, a time point in which about half of the cells came off the dish. The detached subpopulation presented more P2X7 mRNA and protein when compared to the adherent subpopulation, indicating that the subpopulation of cells with more P2X7 expression were more susceptible to ATP (Fig. 3A,B). No differences were found in the mRNA of the other P2X receptors (Fig. 3C), corroborating the importance of P2X7 in the ATP-induced cell death.

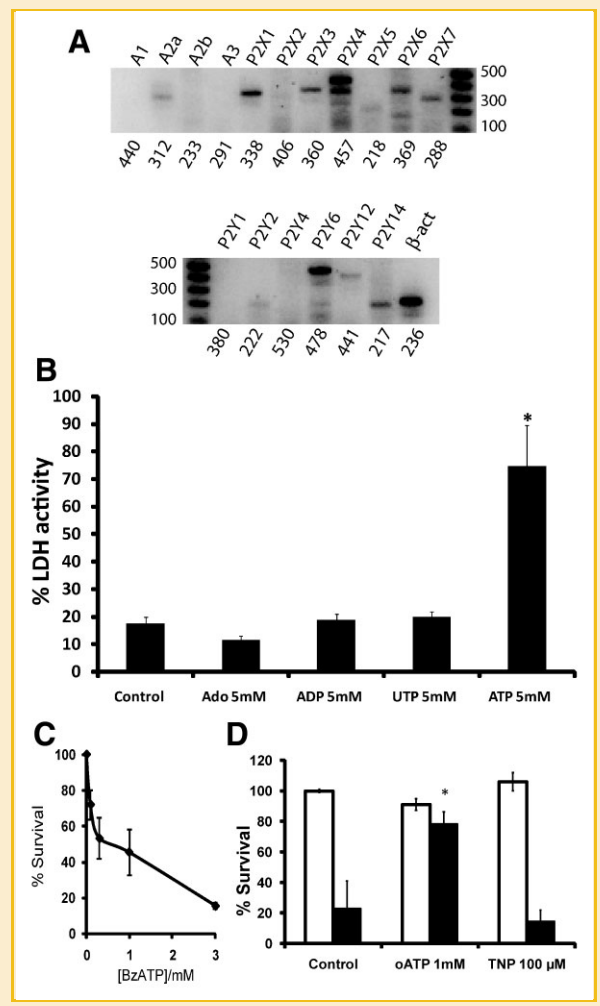

Fig. 2. Pharmacological profile of GL261 cell death: mRNA expression of purinergic receptors in GL261 analyzed by RT-PCR (numbers below indicate the size of the expected PCR product. Representative of three experiments) (A). LDH release from GL261 cultures treated for 2 h with 5 mM of the indicated agonists, $n = 3$ (B). Dose–response curve of BzATP treatment for 2 h measured with MTT assay (C). Effect of a P2X7 antagonist, oATP, and a P2X1-6 antagonist, TNP-ATP, on ATP-induced cell death, white bars represent control cells and black bars are 5 mM ATP-treated cells, $n = 3$ (D). $^{**}P < 0.01$ versus controls and $^{*}P < 0.05$ versus controls with *t*-test.

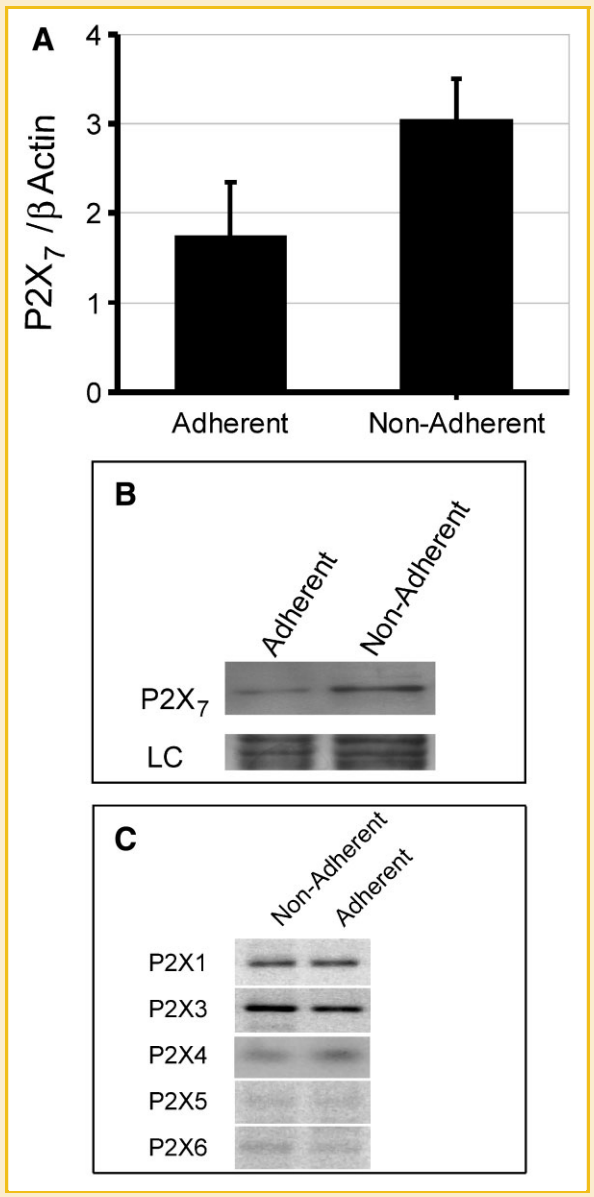

Fig. 3. Expression of P2X7 is higher in a ATP-sensitive sub-population: P2X7 expression analyzed by quantitative RT-PCR (average ± SD, n = 2) (A) and Western blot (B) in cells that came off the plate with 5 mM ATP for 20 min (non-adherent) when compared to the cells that remain attached to the plate (adherent). Loading Control (LC) represents PVDF membrane stained with comassie blue. Comparative expression of the other P2X receptors in adherent and non-adherent cells by RT-PCR (C).

SILENCING OF P2X7 DRASTICALLY REDUCES ATP-INDUCED CELL DEATH

Considering the pharmacological profile of cell death, together with the enrichment of P2X7 receptor in the more sensitive cells, we set out to silence P2X7 receptor with stable shRNA. P2X7 expression, as verified by Western blotting, was reduced by more than 90% in the P2X7 knockdown (KD) cells (Fig. 4A). KD cells presented no detectable morphological alteration when compared to wild type (WT) cells under normal growth conditions (Fig. 4B). Upon ATP treatment, WT cells rounded up and came off the culture plate while KD cells did not present significant alterations in morphology, similarly to WT cells treated with the P2X7 antagonist, oATP (Fig. 4B). Both 3 and 5 mM ATP lead to a significant increase in PI permeabilization within 2 h in WT cells or cells transduced with the non-target shRNA sequence (data not shown) while only a mild increase in PI positive cells was observed with 5 mM ATP in KD cells (Fig. 4B,C). It is important to notice that LDH liberation remained at the basal level in KD cells, even at 5 mM ATP (Fig. 4D) suggesting that KD cells probably open up some pores through which PI can pass, but this is not sufficient for the induction of membrane rupture and release of LDH.

Previously we showed that several glioma cell lines are resistant to ATP-induced cell death [Morrone et al., 2005]. A rat glioma cell line (C6) and two human gliomas cell lines (U138 and U87) which are resistant to 5 mM of ATP as verified by LDH activity (Fig. 5A), presented similar P2X7 receptor expression when compared to GL261 cells (Fig. 5B) suggesting that the presence of P2X7 is not sufficient for ATP-induced glioma cell death.

ATP-INDUCED CELL DEATH WITH NECROTIC BUT NOT APOPTOTIC FEATURES

ATP treatment induced a clear increase in the PI-positive population, without an increase of the annexin V-positive population (Fig. 6A,B), in strong contrast with the positive control, cisplatin (Supplementary Fig. 2A). The lack of apoptosis in response to ATP is confirmed by the absence of caspase 3/7 activity in cells treated with 5 mM ATP for 2 h (Supplementary Fig. 2B) or 24 h (data not shown).

Treatment with ATP lead to a clear shrinkage of the cells, as indicated to the left-shift in the forward scatter analysis of cells treated with ATP (Fig. 6, compare panels D with F) and this shrinkage did not differ between the PI-positive and negative population, suggesting that permeabilization to PI and shrinkage are events that are not causally linked. This is further supported by the observation that P2X7 KD significantly reduced permeabilization to PI but did not significantly affect shrinkage (Fig. 6, compare panels H and F), suggesting that the latter is mediated by another P2 receptor.

These results suggest that permeability to PI, probably due to P2X7 channel to pore transition is an important part of cell death induced by ATP, and that KD of P2X7 reduces the amount of receptor to a level which allows the entry of PI in some cells but does not reach the threshold needed for cell rupture.

DISCUSSION

In the present report we show that ATP induces cell death of the mouse glioma cell line GL261 and that this effect requires the activation of the P2X7 receptor. This is supported by the pharmacological profile, with cell death being induced by concentrations of ATP and BzATP, a P2X7 agonist, in the concentration range described for the mouse P2X7 activation together with the blockage of ATP-induced cell death by oATP, a P2X7 antagonist. Additionally, the lack of toxic effects found by high concentrations of ADP, adenosine or UTP, P1 and P2Y agonists, suggests that the activation of receptors whose mRNA was found in GL261, such as A_{2a}, P2Y2, P2Y6, P2Y12, or P2Y14 are not

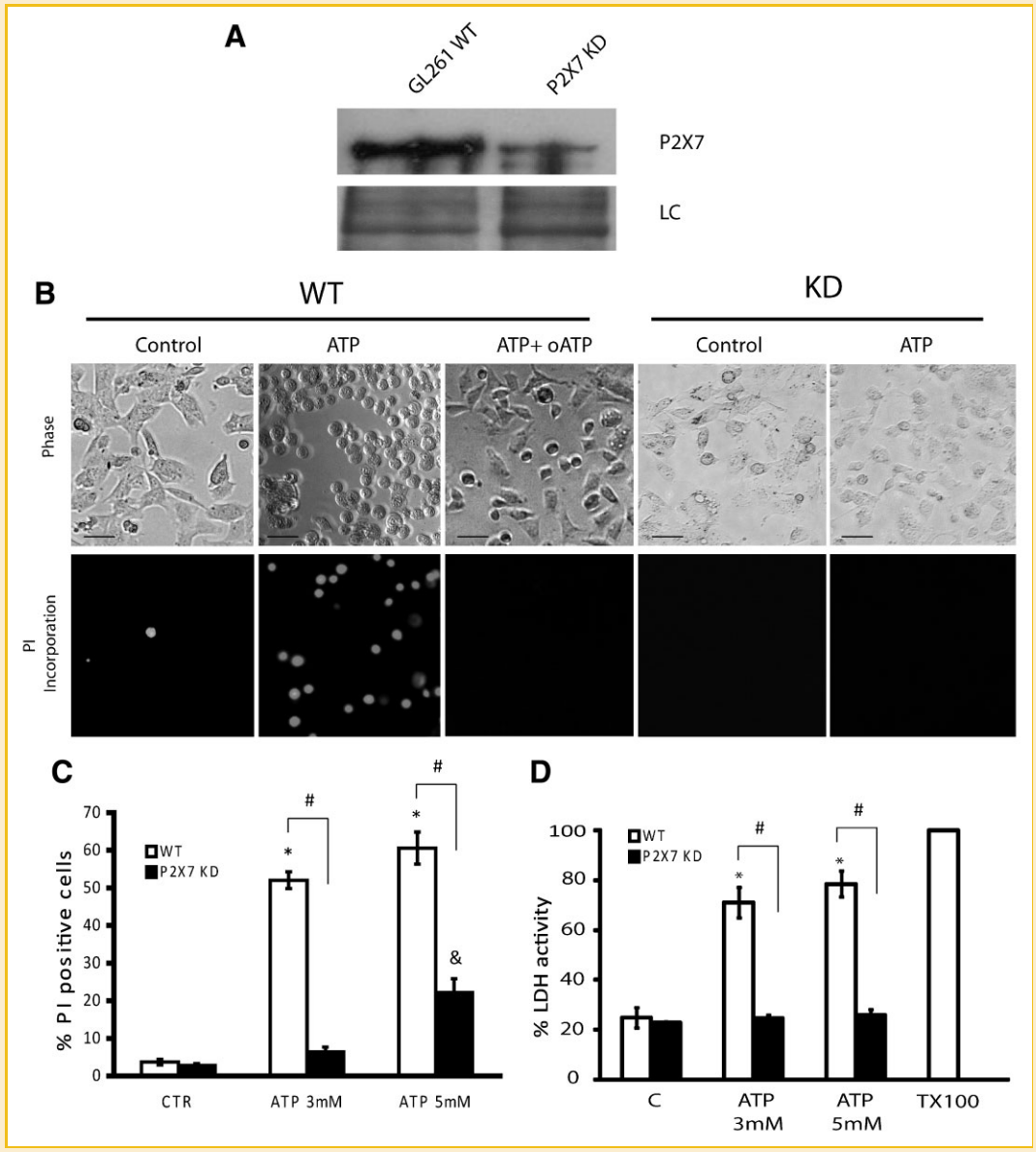

Fig. 4. P2X7 is necessary for ATP-induced cell death in gliomas. Stable silencing of P2X7 via lentiviral-mediated transduction of shRNA reduced P2X7 expression in GL261 as shown by Western blotting (A). Morphological changes and PI incorporation in WT or P2X7 KD cells treated with 5 mM ATP in the presence or not of 1 mM of oATP, Bars represent 50 μm (B). PI positive cells quantified by flow cytometry (C) and LDH liberation (D) of WT or KD of cells treated with 3 or 5 mM of ATP for 2 h. $^{*}P < 0.01$ when compared to untreated, $^{\#}P < 0.05$ and $^{\&}P < 0.01$ when compared to WT cells (one-way ANOVA followed by Tukey's test).

responsible for the toxicity of ATP or its degradation products. Differential expression of P2X7 mRNA and protein in sub-populations with different sensitivities to ATP indicate that the more sensitive sub-population expresses more P2X7, while the expression of other P2X receptors was not positively or negatively selected for. Most importantly, the KD of P2X7 in GL261 produced a cell line resistant to 5 mM ATP, indicating that this receptor is necessary for the ATP-induced cell death in GL261. P2X7 receptor, despite being necessary for ATP-induced cell death in GL261, is not sufficient for ATP-induced cell death in gliomas in general, since other glioma cell lines express similar levels of P2X7 when compared to GL261, but are resistant to 5 mM ATP. This suggests that, in these gliomas, P2X7 is either inactive by mutation or truncation or the expression of accessory proteins that are necessary for mediating the toxic effects of ATP, such as pannexin, is lacking [Pelegrin and Surprenant, 2006].

Cell death induced by activation of P2X7 is strongly dependent on cell type. There are reports of classical apoptosis or necrosis as the predominant cell death mechanism [Coutinho-Silva et al., 2005; Tsukimoto et al., 2005], but, in the majority of cases, a mixed feature of cell death mechanisms was found, mainly with apoptosis and necrosis co-occurring. Since none is dominant, the block of apoptosis generally does not prevent cell death [Auger et al., 2005; Jun et al., 2007]. Even a pseudoapoptotic mechanism was proposed

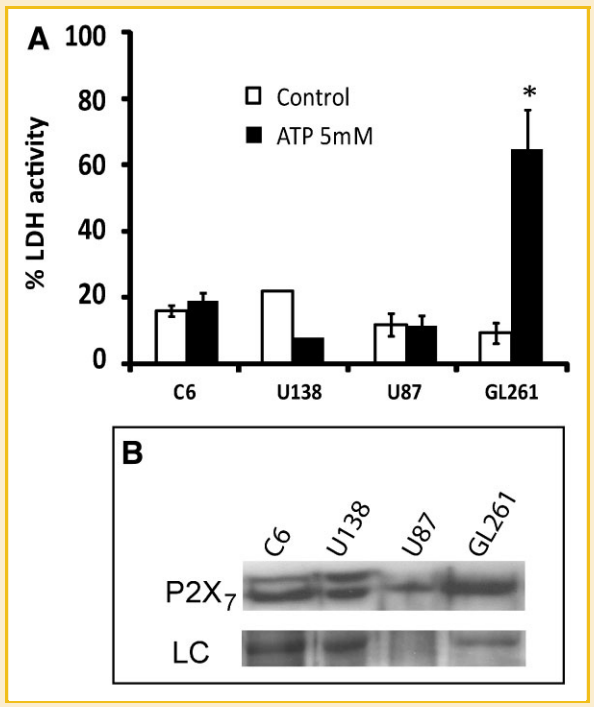

Fig. 5. P2X7 receptor presence is not sufficient to trigger ATP-induced cell death. Rat (C6) or human (U138 and U87) glioma cell lines were treated with 5 mM of ATP for 2 h and LDH activity was measured (A). Level of P2X7 expression was evaluated by Western blotting (B). Loading Control (LC) represents PVDF membrane stained with comassie blue. $^{*}P < 0.05$ versus controls with *t*-test.

for P2X7-mediated cell death, in which cells presented mitochondrial depolarization with swelling, PS flip, membrane blebbing but without subsequent cell death [Mackenzie et al., 2005]. We observed a cell death that presents predominant necrotic features, such as membrane permeabilization and rupture. The observation that ATP-induced some increase in PI positive cells without leading to any LDH liberation in P2X7 KD cell suggests that a threshold of receptor activation has to be reached for cell rupture, but not for P2X7 pore opening and entry of PI [Greenberg et al., 1988].

Apoptosis is normally preceded by cell shrinkage whereas necrosis is preceded by swelling [Okada and Mak, 2004]. We observed cell shrinkage, but that was not accompanied by PS externalization neither by caspase 3/7 activation, suggesting that apoptosis is not the mechanism of ATP-induced cell death in GL261. Additionally, the observation that the rate of cells that shrunk was not different for the PI positive and negative population suggests that these events are not causally linked but rather co-occurring. This is further supported by the shrinkage observed in P2X7 KD cells, but which was not accompanied by a large permeabilization and, most importantly, cell death. Others have observed cell shrinkage induced by P2X7 activation in lymphocytes, but this was accompanied by PS externalization [Taylor et al., 2008; Tsukimoto et al., 2005]. Recently, Takenouchi et al. [2009] reported that activation of P2X7 receptor lead to a decrease in autophagy in microglial cells, through the impairment of lysosomal function. Preliminary data indicate that ATP reduces autophagy in GL261 cells and that P2X7 KD does not significantly affect this reduction induced by ATP, suggesting that autophagic cell death does not play an important role in ATP-induced cell death and that $P2X_7$ does not regulate autophagy in GL261 cells.

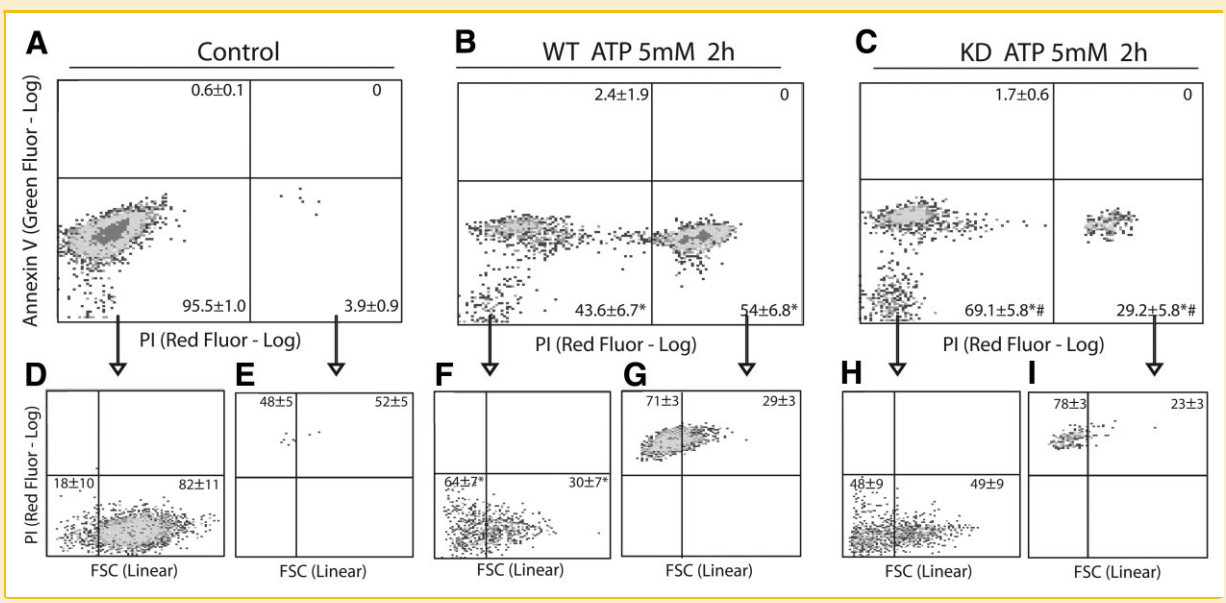

Fig. 6. ATP-induced cell death involves membrane permeabilization, cell shrinkage but not PS externalization. WT or KD cells were treated with 5 mM ATP for 2 h and analyzed by flow cytometry with Annexin V and PI. Panels A–C: Annexin V staining × PI incorporation. Panels D–I: Cell size (Forward Scatter, FSC) plotted against PI incorporation of the indicated populations. Values refer to average of the percentage of cells in each gate of six independent experiments ± SEM. $^{*}P < 0.05$ versus WT control cells and $^{\#}P < 0.05$ versus WT ATP 5 mM (one-way ANOVA followed by Tukey's test).

Several reports point to stem cells as the origin of gliomas, although this is still an area of dispute, mainly due to uncertainties about the promoter activities in the different neural stem cells [Shih and Holland, 2004]. Neural progenitor cells (NPCs) are sensitive to ATP [Delarasse et al., 2009], with several similarities to cell death induced by ATP in GL261, which include: (i) dose–response to ATP and BzATP; (ii) inhibition of ATP-induced cell death by oATP, (iii) time curve of LDH release, (iv) lack of involvement of caspases, and (v) an almost complete dependence of the expression of P2X7, as evidences by the reduction of ATP-induced cell death of NPCs obtained from P2X7 KO mice. On the other hand, there are many differences in ATP-induced death between GL261 and neurons such as protection of the latter but not the former by the P2X1-6 antagonist TNP-ATP [Cavaliere et al., 2004, 2007] and only a mild reduction of death by P2X7 KO in neurons, but a large reduction by P2X7 KD in GL261. Considering that astrocytes are resistant to ATP-induced cell death, these suggests that GL261, at least regarding sensitivity to ATP, are more similar to NPCs than to differentiated CNS cells.

The purinergic system plays a role in the growth of gliomas, which was evidenced by the induction of proliferation of gliomas by ATP in vitro [Morrone et al., 2003] and the extremely low ecto-ATPase activity in gliomas when compared to astrocytes [Wink et al., 2003]. In vivo models showed that expression of ecto-enzymes that degrade ATP alter tumor growth depending of the product of ATP degradation. Presence of apyrase, which degrades ATP to AMP, produced a reduction of C6 rat glioma growth [Morrone et al., 2006] whereas expression of ecto-NPDase2, an enzyme that degrades ATP in ADP, increased tumor size [Braganhol et al., 2009]. This suggests that ATP and the purinergic system is a significant player in the biology of glioma tumors and suggests that ATP is present at the tumor site and may affect survival depending on the sensitivity of the glioma cells to ATP.

GL261 is a well-established glioma cell line that can grow in the brain of mice [Szatmari et al., 2006], suggesting that sensitivity to high concentrations of ATP does not totally abrogate in vivo glioma growth. There are indirect evidences that extracellular ATP reaches levels needed for P2X7 activation in the CNS, since elimination of pathogenic T lymphocytes is higher in wild-type mice when compared to P2X7 KO in experimental autoimmune encephalomyelitis [Chen and Brosnan, 2006]. It is not clear, however, if gliomas experience concentrations of ATP high enough to induce cell death, but the higher ecto-ATPase activity of GL261 (data not shown) when compared to the glioma cells lines that are resistant to ATP [Wink et al., 2003] may suggest that GL261 could use this ATPase activity to protect itself against ATP that may accumulate around the tumor.

P2X7 has been linked to various types of cancer, mainly by correlative observations such as overexpression, found in prostate [Slater et al., 2004a], breast [Slater et al., 2004b], skin [Greig et al., 2003], neuroblastoma [Raffaghello et al., 2006], leukemia [Mackenzie et al., 2005], and thyroid papillary carcinoma [Arcella et al., 2005]. A large multicentric study showed that patients that have gliomas with high expression of P2X7 mRNA present longer survival when compared to patients with gliomas that express less P2X7 mRNA [The-Cancer-Genome-Atlas, 2008] thus supporting the notion that P2X7 presents an anti-tumor effect for gliomas. On the other hand, in neuroblastoma cells, P2X7 activation was shown to lead to an increase in tumor growth through substance P release [Raffaghello et al., 2006].

Tumor growth leads to the production and accumulation of several substances normally not found in healthy tissue and, although the majority of tumor cells evolved to be immune, and even strive, on these substances, the modulation of their response on the tumor cells may lead to molecules or genetic therapies aimed at sensitizing the tumor cells to the naturally occurring molecules, such as ATP, that accumulate around the tumor.

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