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Expression and function of the $P2X_7$ receptor in rat C6 glioma cells

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Abstract

Our results demonstrate the first findings of expression and function of the purinergic $P2X_7$ receptor ($P2X_7R$) in rat C6 glioma cells. $P2X_7R$ mRNA and protein were present in unstimulated C6 cells and were up-regulated by cell exposure to the $P2X_7R$ agonist, 2′,3′-(benzoyl-4-benzoyl)-ATP (BzATP). Activation of $P2X_7R$ in C6 in response to BzATP led to increased mobilization of intracellular calcium $[Ca^{2+}]i$ and formation of large pores. Chronic exposure of C6 cells to BzATP enhanced the expression of pro-inflammatory factors including MCP-1, IL-8 and VEGF. In a scratch-wound migration assay, the $P2X_7R$ was shown to regulate cell mobility. The overall results suggest that $P2X_7R$ activation in C6 is linked with increased pro-inflammatory factors and tumor cell migration.

Keywords: Rat C6 glioma cells; $P2X_7$ receptor; Calcium imaging; Migration; BzATP; OxATP

1. Introduction

Extracellular adenosine triphosphate (ATP) serves important roles in purinergic-mediated signaling as a neurotransmitter and a modulator of cellular functional responses. ATP produces its effects via the activation of P2 receptors comprising the metabotropic P2YR and the ionotropic P2XR receptor families [1,2]. The P2XR family includes subtypes $P2X_1R$–$P2X_7R$ which are coupled to non-selective cationic channels passing Na^+ and Ca^{2+} influx and K^+ efflux. A particularly unique member of the P2XR family is the subtype $P2X_7R$ that, when activated by ATP binding, forms a large pore allowing permeability to large hydrophilic molecules. A number of studies have reported that $P2X_7R$ expression is up-regulated under pathological conditions and can mediate cellular inflammatory responses [3–6].

Gliomas are the most common primary brain tumors in adults and the rat C6 glioma model system has been widely used to elucidate mechanisms underlying the aggressive nature of these brain tumors [7]. Recent evidence suggests that ATP signaling may be involved in glioma development and that P2 receptors might provide novel therapeutic tools in the treatment of brain cancer [8,9]. Sev-

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eral purinergic receptors of the P2YR family are expressed in C6 cells including $P2Y_1$, $P2Y_2$ and $P2Y_{12}$ [10,11] and P2YR have been reported to mediate changes in intracellular Ca^{2+} levels $[Ca^{2+}]i$ following ATP application [11] and to regulate glioma cell proliferation [12]. At present, however, no studies have reported involvement of P2XR in the progression of brain tumors. In this work we document expression and functional responses of the subtype $P2X_7R$ in C6 glioma cells.

2. Materials and methods

2.1. Cell culture

Glioma C6 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells from passage number 39–59 were used in this work. Cells were cultured in Kaighn's modification of Ham's F12 medium (F12K) with 2 mM L-glutamine modified by ATCC to contain 1.5 g/l sodium bicarbonate [13]. The medium was then supplemented with 15% horse serum, 2.5% fetal bovine serum, 0.5 μg/ml fungizone (Invitrogen: GIBCO, Grand Island, NY) and 0.02 mg/ml gentamicin (Invitrogen: GIBCO). Cells were maintained in 100 mm culture dishes (SARSTEDT, Newton, NC) at 37 °C in a humidified 5% CO_2 air atmosphere.

2.2. Calcium-sensitive spectrofluorescence

The methods for preparation of cells for calcium imaging followed published procedures [14]. Briefly, cultured C6 glioma cells were loaded with fura-2 acetoxymethyl ester (fura-2AM at 1 μM; Molecular Probes, Eugene, OR) and pluronic acid (at 1 μM) in normal physiological saline solution (PSS) for 20 min at room temperature (20–22 °C). In some experiments, cells were incubated with the $P2X_7R$ antagonist periodate-oxidized ATP (OxATP, 300 μM for 2 h; Sigma, St. Louis, MO) prior to dye loading. Cells were then washed for 10 min in PSS solution containing (in mM): 126 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES and 10 D-glucose (pH 7.4). In several studies Ca^{2+}-free PSS was used; this solution had the same composition as PSS with the exception that EGTA was added (at 1 mM) with no $CaCl_2$. Coverslips were placed in a perfusion chamber mounted on an inverted microscope (Zeiss, Jena, Germany) and fluorescence was measured through a 40× quartz objective lens. Alternating wavelengths (340/380 nm) of ultraviolet light were applied at 6-s intervals for excitation and fluorescence signals were measured at 510 nm of emission light. Signals were acquired from a digital camera (DVC-1310, DVC Co. Austin, TX) and were processed using an imaging system (Empix, Mississauga, ON, Canada) to determine ratios of the 340 and 380 nm intensities which were used as quantitative measures of fluorescence levels in this work. All studies were done at room temperature.

2.3. Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol (GIBCO-BRL, Gathersburg, MD) and then processed for the first strand complementary DNA (cDNA) synthesis using Moloney murine leukemia virus (M-MLV) reverse transcriptase (GIBCO-BRL). The cDNA products were then amplified by PCR using a GeneAmp thermal cycler (Applied Biosystems, Foster City, CA). Specific sense and antisense primers with the expected product size were: rat $P2X_7R$ sense 5′-AGGAGCCCCTTATCAGCTCT-3′ and rat $P2X_7R$ antisense 5′-CATTGGTGTACTTGTCGTCC-3′ (692 bp); rat monocyte chemoattractant protein-1 (MCP-1) sense 5′-CCTGTTGTTCACAGTTGCTGCC-3′ and rat MCP-1 antisense 5′-TCTACAGAAGTGCTTGAGGTGGTTG-3′ (396 bp); rat interleukin-8 (IL-8) sense 5′-GAAGAT AGATTGCACCGATG-3′ and rat IL-8 antisense 5′-CATAGCCTCTCACACATTTC-3′ (365 bp); rat vascular endothelial growth factor (VEGF) sense 5′-GCTCTCT TGGGTGCACTGGA-3′ and rat VEGF antisense 5′-CA CCGCCTTGGCTTGTCACA-3′ (644 bp); rat β-actin sense 5′-GTGGGGCGCCCCAGGCACCA-3′ and rat β-actin antisense 5′-GTCCTTAATGTCACGCACGA TTTC-3′ (526 bp). PCR conditions were as follows: initial denaturation at 95 °C for 6 min followed by a 25- to 30-cycle amplification program consisting of denaturation at 95 °C for 45 s, annealing at 55–60 °C for 1 min and extention at 72 °C for 1 min. A final extention was carried out at 72 °C for 10 min. β-actin was used as a reaction standard. The amplified PCR products were identified using 1.5% agarose gels containing ethidium bromide (final concentration 0.5 μg/ml) and visualized under ultraviolet light. The intensities of each band were measured by densitometry using NIH Image J 1.37b software (National Institute of Health, Bethesda, MD) and expressed as relative mRNA levels (mRNA levels normalized to β-actin).

2.4. Western blot analysis

Total proteins from the C6 glioma cell line or rat brain tissues were prepared as described previously [15]. Protein samples were analyzed by SDS–PAGE and Western blot analysis [16]. The primary antibodies used were $P2X_7R$ (1:1000, Alomone Labs, Jerusalem, Israel) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:800, Lake Placid, NY). The bound primary antibody was detected by the relevant horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000, Amersham-Pharmacia Biotech, NJ) and an enhanced chemiluminescence kit (ECL,