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Roles of $P2X_7$ Receptor in Glial and Neuroblastoma Cells: The Therapeutic Potential of $P2X_7$ Receptor Antagonists

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Abstract Recently, one of the P2 purinergic receptors, the $P2X_7$ receptor, has been extensively studied in nervous system and important functions have been revealed in both astrocytes and microglia. Stimulation of the receptors induces a sustained and nondesensitized increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). In astrocytes purinergic receptors primarily regulate neurotransmission by inducing gliotransmitters release whereas in microglia the receptors stimulate the processing and release of proinflammation cytokines such as interleukin-1 and are thereby involved in inflammation and neurodegeneration. Thus, $P2X_7$ receptors are considered not only to exert physiological functions but also mediate cell death. $P2X_7$ receptors have also been identified in various cancer cells and in neuroblastoma cells. In these cells, the $P2X_7$ receptor-mediated sustained Ca^{2+} signal is important in maintaining cellular viability and growth. Accordingly, these findings not only lead to a better understanding of roles of the receptor but also prompt the development of more potent, selective and safer $P2X_7$ selective antagonists. These emerging antagonists bring new hope in the treatment of inflammatory-induced neurodegenerative diseases as well as neuroblastoma.

Keyword Astrocytes · N2a neuroblastoma cells · Oxidized ATP · $P2X_7$ receptor · $P2X_7$ receptor antagonists

Introduction

ATP has been well accepted as a neurotransmitter [1] and more recently as a gliotransmitter [2]. ATP plays many important roles in the nervous system, especially in modulating neurotransmission via two families of purinergic receptors, P2Y and P2X. Both P2 receptors are widely distributed in the CNS [1, 3]. P2Y receptors are G-protein coupled receptors consisting of eight subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$. P2X receptors are ionotropic receptors and consist of receptor subtypes: $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, and $P2X_7$. The $P2X_7$ receptor was initially classified as a nonselective, pore-forming P2Z class that induces cytolytic activities in macrophages and other types of cells. Early studies revealed that activation of the P2Z receptor required a high concentration of ATP [3] allowing a bidirectional increase in plasma membrane permeability to molecules as large as 900 Da and induced the release of endogenous molecules [4, 5]. Therefore, the receptor has been suggested to mediate the release of gliotransmitters in the nervous system. The P2Z receptor was cloned from rat brain and showed sequence homology to the P2X receptor and was therefore renamed $P2X_7$ receptor [6]. In the nervous system, $P2X_7$ receptors can be found on microglia [7], both type-1 and type-2 astrocytes [8–10], and presynaptic terminals of neurons [11].

$P2X_7$ Receptor-Mediated Ca^{2+} Influx in Astrocytes Using Specific Agonists and Antagonists

The ATP analogue 2'-,3'-*O*-(4-benzoylbenzoyl)-ATP (BzATP) was originally introduced as a photoaffinity label for ATPase [12] and was shown to be a potent agonist for P2 receptors in the transform fibroblasts [13]. Since then, BzATP has been shown to be extremely potent at the $P2X_7$ receptor and is widely used as its selective agonist [5, 14]. In addition, several agonists have been shown to activate $P2X_7$ receptor with distinct potency [1, 3, 14–16]. Periodate-oxidized 2',3'-dialdehyde ATP (oATP) is a

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Schiff-base forming reagent commonly used as a selective $P2X_7$ receptor antagonist. Effective inhibition of $P2X_7$ receptors requires a concentration of 100–600 μM and a 2-h pretreatment [17]. Using both agonist (BzATP) and antagonist (oATP), Ballerini et al. [8] were the first to show $P2X_7$ receptor-mediated Ca^{2+} influx from extracellular space in astrocytes demonstrating activation and function of $P2X_7$ receptor. Further, in RBA-2 type-2-like astrocytes, BzATP was much more potent than ATP, adenosine 5'-*O*-(3-thiotriphosphate), and 2-methylthioadenosine triphosphate, whereas ADP, UTP, α,β methylene-ATP, and β,γ-methylene-ATP were ineffective in raising intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) [10]. BzATP was further shown to stimulate a sustained and nondesensitized increases in $[Ca^{2+}]_i$ in RBA-2 type-2-like astrocytes [18]. Using similar methods, $P2X_7$ receptor-mediated Ca^{2+} influx was also identified in rat cortical type-1 astrocytes [9]. Additionally, electrophysiological studies revealed that activation of $P2X_7$ receptor did not induce desensitization in HEK cells overexpressed with $P2X_7$ receptors cloned from rat brain [5, 6]. Thus, $P2X_7$ receptors may exert important physiological functions downstream of Ca^{2+} signaling in astrocytes.

$P2X_7$ Receptor Induces Pore Formation and Gliotransmitter Release in Astrocytes

The most unique feature of $P2X_7$ receptors is the prolonged agonist stimulation-induced pore formation that allows uptake of molecules as large as 900 Da. The phenomenon can be assayed by uptake of fluorescent dyes such as Lucifer Yellow and Yo-Pro-1. Pore formation has been shown to be essential for the $P2X_7$ receptor-mediated IL-1 release in human monocytes [19]. Based on permeability, the astrocytic glutamate release was thought to be mediated through $P2X_7$ receptors directly [20]. Pelegrin and Surprenant [21] showed that pannexin-1, a gap junction hemichannel protein abundantly express in the brain [22], was involved in pore formation of $P2X_7$ receptor. Lo et al. [18] observed a 30- to 60-min delay of BzATP-induced Yo-Pro-1 uptake as compared with the BzATP-induced Ca^{2+} influx in RBA-2 astrocytes but not in $P2X_7$ receptor-transfected HEK cells. RBA-2 astrocytes do not express pannexin family of gap channel proteins but do express protein from connexin family of gap junction proteins such as: Cx30, Cx32, and Cx43 (unpublished data). Additionally, the ATP [23] and glutamate [24] release from astrocytes has been identified to mediate through connexin hemichannel. Thus, connexin may also be involved in pore formation in astrocytes.

Multiple mechanisms are involved in gliotransmitter release from astrocytes, $P2X_7$ receptor being one of them. Initially, activation of $P2X_7$ receptor was shown to induce purine release from astrocytes [8]. Duan et al. [20] later revealed that activation of the $P2X_7$ receptor-mediated glutamate release directly. They found that the $P2X_7$ receptor selective agonist BzATP stimulated inward current of primary astrocytes more potent than ATP-induced permeability to glutamate and aspartate. Furthermore, the BzATP stimulated [^{3}H]aspartate release, stimulation of inward current and release of glutamate were all blocked by the $P2X_7$ receptor selective antagonist, oATP. More recently, through the use of genetically modified 1321N1 astrocytoma cells and spinal cord astrocytes derived from the neonatal Cx43- and $P2X_7$ receptor-null mice, Suaducani et al. [25] provided strong evidence showing that $P2X_7$ receptors, but not Cx43 hemichannels, were the sites of ATP release.

Type-2 astrocytes are GFAP- and A2B5-positive, process-bearing astrocytes, and their role may be to surround the synapses and modulate extracellular neurotransmitter concentration [26]. Using BzATP and oATP, activation of $P2X_7$ receptors was shown to stimulate [^{3}H]GABA release through a Ca^{2+}-independent and Cl^-/HCO_3^--dependent mechanism, regulated by protein kinase C, cAMP-dependent protein kinase, mitogen-activated protein kinase kinases/extracellular signal-regulated kinases, and phospholipase D in RBA-2 type-2-like astrocytes [27]. Similarly, the ATP-induced ATP release from primary cortical astrocytes was also found to be Ca^{2+}-independent and was inhibited by nonselective anion channel blockers [28]. In addition, the ATP-stimulated [^{3}H]GABA release was almost completely blocked by a high concentration of extracellular Mg^{2+} [27]. The divalent ions have been shown to inhibit the whole-cell currents and uptake of Yo-Pro-1 in HEK293 cells expressing the rat $P2X_7$ receptor [5, 29]. This inhibition has been considered a specific property of the $P2X_7$ receptor suggesting that ATP^{4-} may be the ligand for the receptor. Taken together, the $P2X_7$ receptor may modulate neurotransmission by controlling the release of gliotransmitters from astrocytes through multiple mechanisms.

$P2X_7$ Receptors Mediate Cell Death in the Nervous System

Activation of P2Z/$P2X_7$ receptors is known to mediate cytolysis [6]. The proinflammatory cytokine, interleukin (IL)-1 plays a significant role in neuronal death following pathological insults [30], and $P2X_7$ receptors can induce the processing and release of IL-1β in the immune responsive cells [31]. Using $P2X_7$ receptor knockout mice, $P2X_7$ receptors were confirmed to be involved in maturation and release, but not the synthesis of IL-1β [32]. In the nervous system, microglial cells were found to express the $P2X_7$ receptor and to be exquisitely sensitive to ATP-mediated cytotoxicity [33]. $P2X_7$ receptor-mediated IL-1β secretion was identified in microglia [34], and the receptor-induced

sustained increases in $[Ca^{2+}]_i$ was shown to play a pivotal role in potentiation of the secretion [35]. Mechanistic studies further revealed that the extracellular ATP triggers fast maturation and release of intracellularly accumulated IL-1β by activating the IL-1β-converting enzyme/caspase 1 in microglia [36]. Taken together, it is clear that $P2X_7$ receptors are involved in IL-1-mediated inflammation and neurodegeneration in the nervous system. Thus, the selective antagonists of $P2X_7$ receptors are emerging as a new therapeutic strategy.

$P2X_7$ Receptors are Important in Maintenance of Cell Viability of Neuroblastoma Cells

The physiological function of $P2X_7$ receptor in neuronal or neuronal-like cells remains elusive. $P2X_7$ receptor signaling has been identified in NG108-15 [37] and SH-SY5Y [38] neuroblastoma cells. Stimulation of $P2X_7$ receptors supported proliferation in human neuroblastoma cells [39]. However, $P2X_7$ receptors were also found to trigger the death of retinal cholinergic cells thereby controlling the total number, the local density, and spacing of neurons [40]. The expression and function of $P2X_7$ receptor was shown to correlate with the severity of B cell chronic lymphocytic leukemia [41]. An early study demonstrated that transfection of $P2X_7$ cDNA into the $P2X_7$ receptor lacking lymphoid cells sustained the proliferation in a serum-free medium [42]. In addition, $P2X_7$ receptors were absent from normal, cancer-free prostate epithelium obtained from young men, whereas $P2X_7$ receptors was present in every case of 116 confirmed prostate cancers regardless of patient age [43]. Thus, the $P2X_7$ receptor may support proliferation of the certain cancer and neuroblastoma cells.

Functional Inhibition of $P2X_7$ Receptors by Selective Antagonists Induce Neuronal Differentiation of N2a Neuroblastoma Cells

Although $P2X_7$ receptors mediate neurotransmission via astrocytes, they are also known to be involved in the release of proinflammatory cytokines by microglia suggesting a role in inflammation. Recently, inhibition of $P2X_7$ receptors was found to promote axon outgrowth and branches of hippocampal neurons [44]. N2a neuroblastoma cells are known to extend neurite growth in response to various differentiation agents. Retinoic acid (RA)-induced neurite outgrowth and neuronal marker expression were associated with decreases in expression and function of $P2X_7$ receptors [45]. Interestingly, neurite outgrowth was also induced through functional inhibition of $P2X_7$ receptors by the selective antagonists, oATP or brilliant blue G (BBG) or A438079, or knockdown $P2X_7$ receptor expression by siRNA. To elucidate the possible mechanism, the levels of basal $[Ca^{2+}]_i$ were measured. RA, oATP, and knockdown $P2X_7$ receptor all decreased basal levels of $[Ca^{2+}]_i$. Simply culturing N2a cells in a low Ca^{2+} medium induced a twofold increase in neurite length. Inhibition of $P2X_7$ receptors or application of apyrase to degrade extracellular ATP decreased cell viabilities, suggesting that endogenous ATP release-activated $P2X_7$ receptors may be important in maintaining cell viability of N2a cells [45]. Recently, endogenous ATP release was also found to activate $P2X_7$ receptors channel in mouse astrocytes [46]. Taken together, these results imply that the $P2X_7$ receptor may be activated under physiological condition via endogenous ATP release. Furthermore, Ca^{2+}/calmodulin-dependent kinase II signaling cascade has been shown to mediate the $P2X_7$ receptor-dependent inhibition of neuritogenesis in N2a cells [47].

Inhibition of $P2X_7$ Receptor is New Therapeutic Strategy

Because of the association with inflammation and neuropathic pain, a great effort has been made to develop more potent and highly selective $P2X_7$ receptor antagonists [48, 49–53]. Treatment of $P2X_7$ antagonists to chronic experimental autoimmune encephalomyelitis (EAE) also reduced demyelination and ameliorated the associated neurological symptoms [54]. BBG and the newly developed $P2X_7$ receptor selective antagonist A438079 [51] were shown to inhibit $P2X_7$ receptors with nanomolar affinity. $P2X_7$ receptors have also been identified in spinal cord neuron [55]. In a peritraumatic zone spinal cord injury model, exposure to ATP led to high-frequency spiking, irreversible increases in cytosolic calcium and cell death; however, treatment with antagonists significantly diminished cell death and improved functional recovery [55]. Recent evidence showed that administration of the $P2X_7$ antagonist, BBG, 15 min after spinal cord injury, reduced spinal cord anatomic damage and improved motor recovery, indicating that BBG not only protected spinal cord neurons from purinergic excitotoxicity, but also reduced local inflammatory responses [56].

We have recently shown that $P2X_7$ receptor antagonists, oATP, BBG, and A438079, all stimulated neurite outgrowth of N2a neuroblastoma cells. Nevertheless, the BBG- and A438079-stimulated neurites were shorter than those stimulated by oATP [45]. In addition, oATP, but not BBG, inhibited cell proliferation and altered cell cycle progression. This may be because, while BBG and A438079 are reversible antagonists [51, 57], oATP inhibited the $P2X_7$ receptor-mediated currents irreversibly [6] and affect other $P2X_7$ receptor-independent mechanisms [58, 59]. Therefore, effects of oATP on inhibition of proliferation and alteration of cell cycle progression of N2a neuroblastoma cells may be mediated through both $P2X_7$-dependent and -independent mechanisms.

Concluding Remark

$P2X_7$ receptors are ATP-gated ion channel. In astrocytes, activation of these receptors induces a sustained and nondesensitized Ca^{2+} influx. Activation of $P2X_7$ receptor requires a high concentration (≥1 mM) of extracellular ATP and endogenous ATP release from astrocytes [46], and N2a neuroblastoma cells [45] can activate the receptors in the absence of ligand. Thus, $P2X_7$ receptors can be activated under physiological condition to mediate important functions such as neurotransmission and regulation of cell growth. It is also well accepted that $P2X_7$ receptors play a key role in IL-1 processing and secretion in immune responsive cells and is thus involved in inflammatory responses and cell death [60]. In addition, $P2X_7$ receptors play a role in the growth of many cancer cells. Researchers found that inhibition of $P2X_7$ receptors is linked to reducing spinal cord injury [55, 56] and ameliorated neurological symptoms with EAE [54]. These findings suggest novel therapeutic strategy utilizing $P2X_7$ receptor antagonists. The development of more potent, highly selective, and much safer $P2X_7$ receptor antagonists will bring new hope in the treatment of the neurodegenerative diseases.

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