Roles of the $P2X_7$ receptor in C6 astroglioma: in vitro and in vivo studies

by

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Abstract

The purinergic $P2X_7$ receptor ($P2X_7R$) is an ionotropic adenosine triphosphate (ATP) receptor which is closely linked with pathological conditions in the central nervous system (CNS). Gliomas are the most common primary brain tumors with presently no cures. The roles of the $P2X_7R$ in these diseases have not been previously studied and in this work, I have used the rat C6 glioma as an experimental model system to investigate expression and function of the $P2X_7R$ *in vitro* and *in vivo*.

The *in vitro* study has examined expression of the $P2X_7R$ in C6 cells and the involvement of this receptor in mediating cell functional responses. C6 glioma cells were found to express the $P2X_7R$ at both mRNA and protein levels. The $P2X_7R$ agonist, 2', 3'-(benzoyl-4-benzoyl)-ATP (BzATP) induced an increase in intracellular Ca^{2+} concentration, an effect which was largely inhibited by periodate-oxidized ATP (OxATP), an irreversible $P2X_7R$ antagonist. BzATP treatment of C6 cells also resulted in ethidium bromide dye uptake indicating pore formation was induced by $P2X_7R$ activation. Chronic exposure of C6 cells to BzATP showed up-regulation of several pro-inflammatory factors including the chemokines monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) and the angiogenic factor vascular endothelial growth factor (VEGF) suggesting the $P2X_7R$ in C6 cells is involved in mediating inflammation in tumors. In addition, BzATP treatment was found to enhance wound-induced cell migration, an effect which was inhibited in the presence of OxATP, or another $P2X_7R$ antagonist, Brilliant Blue G (BBG).

The *in vivo* study examined whether pharmacological modulation of $P2X_7R$ with

BBG altered tumor growth. C6 glioma cells were implanted into the striatum of rat brain and *in situ* $P2X_7R$ expression was shown to be associated with glioma cells and resident microglia. Preliminary results have indicated that inhibition of $P2X_7R$ leads to a reduced volume of brain tumors formed by transplanted C6 cells.

The overall results from this study demonstrate the novel finding that C6 glioma cells express functional $P2X_7R$ and suggest pharmacological modulation of the $P2X_7R$ could serve as an effective strategy to inhibit the development and progression of brain tumors.

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List of Abbreviations

AD	Alzheimer's disease
Aβ	amyloid-beta
2-AG	2-arachidonoylglycerol
AP-1	activator protein-1
ATP	adenosine triphosphate
BBG	Brilliant Blue G
β-gal	beta-galactosidase
BzATP	2', 3'-(benzoyl-4-benzoyl)-ATP
$[Ca^{2+}]i$	intracellular calcium concentration
CNS	central nervous system
CNTF	ciliary neurotropic factor
COX-2	cyclooxygenase-2
CT-1	cardiotrophin-1
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
EtBr	ethidium bromide
F12K	Kaighn's modification of Ham's F12 medium
GBM	glioblastoma multiforme
GFAP	glial fibrillary acidic protein
IL-1β	interleukin-1β

IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
iNOS	inducible nitric oxide synthase
IP3	inositol triphosphate
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
M-MLV	moloney murine leukemia virus
MCAO	middle cerebral arterial occlusion
MCP-1	monocyte chemoattractant protein-1
MG	microglia
MMP-9	matrix metalloprotease-9
MP	macrophage
NF-κB	nuclear factor-κB
NO	nitric oxide
OSM	oncostatin-M
OxATP	periodate-oxidized ATP
P2XR	P2X receptor
P2YR	P2Y receptor
PGE_2	prostaglandin E_2
ROI	reactive oxygen intermediates
RT-PCR	reverse-transcriptase polymerase chain reaction
SEM	standard error of mean

SOC	store-operated channels
SP	substance P
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
VEGF	vascular endothelial growth factor

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1. Introduction

1.1. P2 receptors: general

Adenosine triphosphate (ATP) is not only a cellular energy source but also a purinergic ligand with important signaling roles as a neurotransmitter and as a modulator of cell functions. ATP produces its effects via the activation of P2 receptors comprising the metabotropic P2Y receptor (P2YR) family and the ionotropic P2X receptor (P2XR) family (Erb, 2006; Illes, 2004).

The P2Y receptors are G-protein-coupled and linked to inositol triphosphate (IP3)-mediated release of calcium (Ca^{2+}) from intracellular endoplasmic reticulum (ER) stores. Depletion of Ca^{2+} from ER stores can subsequently trigger entry of Ca^{2+} through store-operated channels (SOC) located in plasma membrane (McLarnon, 2005). To date, eight P2Y receptor subtypes have been cloned in mammals: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ (Fries, 2006; White and Burnstock, 2006).

The ionotropic P2XR family is coupled to non-selective cationic channels allowing influx of Na^+ and Ca^{2+} and efflux of K^+. To date, seven distinct P2XR subtypes have been identified at the molecular level and named $P2X_1R$-$P2X_7R$. An important consequence of activation of P2XR is cell depolarization, which can serve as a modulatory factor in shaping cellular responses (North, 2002). A particularly unique member of the P2XR family is the subtype $P2X_7$ receptor ($P2X_7R$), which is described in considerable detail in section 1.2.

1.2. $P2X_7$ receptors

1.2.1. The structure of the $P2X_7R$

The $P2X_7R$ was first described by Buisman et al. (1988) and labeled P2Z receptor (Di Virgilio, 1995), until its molecular identification. The full length cDNAs of the $P2X_7R$ were first cloned from a rat brain cDNA library. The $P2X_7$ subunit is 595 amino acids long, having 35-40% homology with the other six members of the P2XR family (Surprenant et al., 1996). Its structure is basically similar to the residual P2X receptor subunits, having two transmembrane domains (M1, M2), a large extracellular domain and N- and C-terminal residues both on the cytoplasmic side (Surprenant et al., 1996). The extracellular loop contains 10 cysteine residues that can form disulfide bonds and a sequence stretch (aa 170-330) likely containing six-stranded β-pleated sheets (Freist et al., 1998). This region has been proposed to host the ATP binding site (Gu et al., 2004). The main structural distinctive feature of the $P2X_7R$ is that its intracellular carboxy terminal domain is much longer (239 amino acids) than those of other P2XR subunits (27-129 amino acids). It is now believed that the long C-terminal tail harbors multiple potential protein and lipid interaction motifs (Denlinger et al., 2001). The representative membrane topology of the $P2X_7R$ subunit is presented in Figure 1-1.

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Figure 1-1. Structure of the $P2X_7R$: membrane topology
The full-length and truncated (ΔC) splice variant of the $P2X_7R$ are shown. The truncated form lacks almost the entire COOH tail (249aa, green, red and short black traits) but bears an extra 18aa (light blue trait). TNFRI homology domain (green) and putative LPS-binding region (red) are shown. Cysteine residues forming putative disulfide bridges are also shown (Ferrari et al., 2006).

Following the cloning and expression of the rat $P2X_7R$, the genes encoding the human (Rssendren et al., 1997), mouse (Chessell et al., 1998) and *Xenopus laevis* (Paukert et al., 2002) $P2X_7R$ were also identified, having 80%, 85% and 50% amino acid sequence homology with the rat receptor, respectively. It is now also clear, that unlike other P2XR subunits, the $P2X_7$ subtype does not hetero-oligomerize and functions only in homo-oligomeric form (Torres et al., 1999), most likely as a homo-trimer.

The activation of $P2X_7R$-gated ion channels leads to two distinct responses depending on the exposure time of the agonist. Following a single brief ATP application, a non-selective inward cationic current can be recorded which is similar to inward currents caused by the activation of homo- or hetero-oligomeric assemblies of other P2X receptor subunits, although with different deactivation kinetics. Upon repeated or prolonged application, the opening of a membrane pore can be detected which allows permeability to high molecular weight molecules and ions up to the size of 800 Da (Surprenant et al., 1996). The pore formation is characterized by the uptake of high molecular weight fluorescent dyes such as ethidium bromide, Lucifer yellow or YO-PRO. Pore activation is followed by cytoskeletal rearrangement such as membrane blebbing (Virginio et al., 1997, 1999a), which can eventually lead to apoptosis in immune cells. The long intracellular C-terminal domain has been shown to be instrumental for the pore-forming property of the $P2X_7R$ (Adriouch et al., 2002; Surprenant et al., 1996). Moreover, a lipopolysaccharide (LPS)-binding site has also been identified close to the carboxy terminus of the receptor (Denlinger et al., 2001), whereby the receptor could translate inflammatory signals to signal transduction

events.

1.2.2. The pharmacological properties of the $P2X_7R$

The pharmacological phenotype of the $P2X_7R$ is relatively well established and distinguishable from other members of the P2XR family. The main properties of the $P2X_7R$ are summarized below (Anderson and Nedergaard, 2006; Duan and Neary, 2006; North, 2002; Sperlagh et al., 2006).

1) A relatively low affinity of the $P2X_7R$ for ATP. The $P2X_7R$ generally requires higher concentrations of ATP for activation, in excess of 100 μM, compared to that required by other members of the P2 receptor family.
2) 2', 3'-(benzoyl-4-benzoyl)-ATP (BzATP), the most commonly used $P2X_7R$ agonist, is 10-30 times potent than ATP. Although BzATP also activates other P2X receptors, a higher potency of BzATP than that of ATP is often taken as evidence of $P2X_7R$ involvement.
3) The effect of ATP and BzATP is potentiated by reducing the concentration of extracellular divalent cations (Ca^{2+} or Mg^{2+}). This property indicates that ATP^{4-} may be the active ligand. Since most of extracellular ATP is complexed with Mg^{2+} or Ca^{2+} in normal physiological media, the proportion of ATP^{4-} in total added ATP is low and relatively high levels of ATP are required to activate the $P2X_7R$. It is also possible that the divalent ions bind on the receptor and exert allosteric inhibition.

4) Relatively specific antagonists that can be used for identifying the $P2X_7R$ include periodate-oxidized ATP (OxATP), Brilliant Blue G (BBG) and KN-62. OxATP, a commonly used antagonist that irreversibly blocks $P2X_7$ receptors, is used at concentrations of 0.1-0.3 mM. OxATP is a slowly equilibrating antagonist so pre-treatment is required to allow enough time for blockade (Murgia et al., 1993). Another compound, BBG is a potent, noncompetitive antagonist displaying remarkable selectivity towards $P2X_7$ receptors in the nanomolar range, having IC_{50} values of about 10 nM at the rat $P2X_7R$ (Jiang et al., 2000). A third antagonist which may be useful for the pharmacological identification of $P2X_7$ receptors is KN-62, a selective antagonist at the human, but not the rat, $P2X_7R$ (Gargett and Wiley, 1997).

1.2.3. $P2X_7$ receptors in brain: expression and functions

1.2.3.1. Expression of $P2X_7$ receptors in different cell types in brain

Initial studies of the $P2X_7R$ distribution in brain concluded that $P2X_7$ immunoreactivity was localized only to immune cells and reactive glial populations in infarct-surrounding regions following middle cerebral arterial occlusion (MCAO) (Collo et al., 1997). This outlook began to change in the late 1990s when several reports of $P2X_7R$ expression in neurons were published (Sperlagh et al, 2006). However, substantial controversy over the use of available antibodies has prompted some to conclude that neurons are actually devoid of the $P2X_7R$ (Anderson and Nedergaard, 2006). Therefore, it appears to be premature to draw final conclusions as

to the neuron-specific localization of $P2X_7R$.

As noted above, glial cells have been thought to be the major cell types expressing $P2X_7R$ in the brain. According to current view, the function of glia is far more than a simple support for the neuronal network. Glial cells play an active role in information processing, controlling the homeostasis of the neuronal microenvironment, the development and differentiation of neural cells and the host-defense response. The main glial cell types of the nervous system are astrocytes, oligodendrocytes, and microglia. To fulfill their diverse roles, glial cells have to communicate with each other and with neurons. For this purpose, they utilize their own signaling systems and the $P2X_7R$ seems to have a pivotal role in these processes.

Astrocytes express mRNA for the $P2X_7R$ (Sperlagh et al., 2006). The $P2X_7R$ immunoreactivity is present on the cell surface of human fetal astrocytes (Narcisse et al., 2005) and is co-localized with astrocytic markers in acutely isolated and cultured astrocytes (Panenka et al., 2001). Microglia originate from monocyte/macrophage precursors and are regarded as the major immunocompetent cell type in the brain. It has been known for a considerable time that microglia express both ionotropic and metabotropic receptors (Norenberg et al., 1994), and the presence of the "pore-forming" ATP receptor was also described before the molecular identification of the $P2X_7R$ (Ferrari et al., 1996). The expression $P2X_7R$ transcripts and of the corresponding protein has been confirmed later both in cultured microglia (Collo et al., 1997; Ferrari et al., 1997a) and in microglia activated following middle cerebral artery occlusion (Collo et al., 1997).

1.2.3.2. Functional roles of $P2X_7$ receptors in glia

Astrocytes:

The astroglial $P2X_7R$ participates in a wide array of normal and pathological functions in brain. The primary intracellular signal following the activation of the $P2X_7R$ is a sustained elevation of intracellular Ca^{2+} caused by Ca^{2+} influx from the extracellular space through the receptor-ion channel complex (Ballerini et al., 1996). This Ca^{2+} signal is then translated to various signal transduction pathways conveying diverse functions. However, it is noteworthy that some of the pathways are not dependent on Ca^{2+}. Thus, $P2X_7R$ activation seems to be a common trigger for astrocytic transmitters and modulators to enter the extracellular space either by Ca^{2+}-dependent or by Ca^{2+}-independent signaling pathways.

It has been well known that astrocytes participate in neuroinflammation underlying a variety of CNS insults including trauma, ischemia and neurodegeneration. Inflammatory stimuli rapidly induce the proliferation and hypertrophy of glial cells, a process called reactive gliosis, and respond with the expression and production of inflammatory cytokines, chemokines and other mediators. $P2X_7$ receptors are involved in these regulatory pathways. For example, activation of the $P2X_7R$ promotes the expression of monocyte chemoattractant protein-1 (MCP-1), a critical factor in the early monocyte infiltration during the neuroinflammatory process (Panenka et al., 2001). Furthermore, phosphorylation of ERK1, ERK2 and p38 MAP kinase is increased; these have determinant roles in the commitment of the cells to apoptosis (Panenka et al., Wang et al., 2003). The phosphorylation of ERK1/2 is mediated through a cellular pathway that is dependent

on both Ca^{2+} and the expression of other intracellular signaling proteins, i.e., Pyk2, c-Scr, IP3K and MEK1/2, respectively (Gendron et al., 2003). Other signaling proteins regulated by the $P2X_7R$ include phospolipase D (PLD) (Sun et al., 1999), transforming growth factor-β1 (TGF-β1) (Wang et al., 2003) and protein kinase B (Akt) (Jacques-Silva et al., 2004). $P2X_7$ receptors have been suggested to mediate the effect of ATP to potentiate interleukin-1β (IL-1β)-induced expression of nuclear factor-κB (NF-κB) and to promote activator protein-1 (AP-1) protein expression (John et al., 2001). In addition, a recent study revealed that the activation of the $P2X_7R$ attenuates LPS-induced tumor necrosis factor-α (TNF-α) release from primary cortical astrocytes (Kucher and Neary, 2005); this contrasts with corresponding data in microglia, where $P2X_7$ receptors stimulate the production of this pro-inflammatory cytokine (Suzuki et al., 2004).

Microglia:

Microglia are the resident immune cells in the brain and they help maintain brain homeostatic mechanisms and detect changes in the brain microenvironment. In these functions, microglia can be considered as surveillance cells of the brain (Kreutzberg, 1996). Microglia can be rapidly activated in response to pathological signals such as ischemia and undergo morphological changes transforming the resting ramified cells to an ameboid form with phagocytic activity, proliferation and production of a wide array of inflammatory mediators. Microglia express $P2X_7$ receptors which convey functions analogous to those of other cells of the monocyte/macrophage lineage upon inflammatory stimuli. Primary microglial cultures and immortalized microglial cell lines respond to ATP and BzATP stimulation with an inward current (Chessell et al.,

1997; Haas et al., 1996; Visentin et al., 1999), membrane depolarization, a sustained increase in intracellular Ca^{2+} levels ($[Ca^{2+}]i$) (Ferrari et al., 1996), and the uptake of dyes (Chessell et al., 1997; Ferrari et al., 1996). Activation of the $P2X_7R$ in microglia stimulates a host of intracellular signaling pathways and modulates cellular responses such as production of inflammatory and neurotoxic compounds.

In microglia, $P2X_7R$ is considered as a key player in IL-1 processing and release (Ferrari et al., 2006). The mechanism underlying ATP-dependent IL-1β maturation and release involves an outwardly directed K^+ conductance and the activation of caspase-1 responsible for the cleavage of pro-IL-1β to the mature, 17kDa form (Sanz and Di Virgilio, 2000). ATP is known to be a full stimulus to induce TNF-α production via a Ca^{2+}-dependent, ERK/JNK/p38 signaling pathway (Hide et al., 2000; Suzuki et al., 2004). In addition, high concentrations of ATP induce inducible nitric oxide synthase (iNOS) mRNA expression and increases nitric oxide (NO) production from rat microglia (Ohtani et al., 2000), an effect likely mediated by the $P2X_7R$. ATP and BzATP also promote generation of reactive oxygen intermediates (ROI), in particular superoxide radicals, dependent on extracellular Ca^{2+}; both OxATP and BBG prevent this effect. The actions of ATP are mediated by the p38MAPK pathway (Parvathenani et al., 2003). 2-arachidonoylglycerol (2-AG) is one of the most abundant endogenous cannabinoid ligands (endocannabinoids) and plays a critical role in neuroinflammation. The activation of the $P2X_7R$ using high concentration of ATP or BzATP markedly increases 2-AG production in primary microglial cultures (Witting et al., 2004). Finally, activated microglia can also provide neuroprotection by releasing trophic factors such as plasminogen (Inoue et al., 1998). This neurotrophic

factor is known to promote the development of mesencephalic dopaminergic neurons and neurite outgrowth from explants of neo-cortical tissue.

1.2.3.3. The pathological roles of the $P2X_7R$ in brain

As mentioned in section 1.2.2, $P2X_7R$ generally require higher concentration of ATP for activation and this characteristic could indicate that activation of the $P2X_7R$ is associated with elevated levels of extracellular ATP. A number of pathological events are known to stimulate ATP release. Such pathological signals include hypotonic (Wang et al., 1996) and mechanical stimuli (Coco et al., 2003; Verderio and Matteoli, 2001), energy deprivation (Juranyi et al., 1999) and inflammatory signals such as bacterial lipopolysaccharide (LPS) (Ferrari et al., 1997b), concanvavalin-A (Filippini et al., 1990), or IL-1β (Sperlagh et al., 2004). In addition, taking into account that the free ATP concentration within living cells under the normal metabolic rate is in the millimolar range, cellular damage is hypothesized to result in very high regional levels of ATP.

The up-regulation of the $P2X_7R$ has been observed in a number of pathological models including energy deprivation (Cavaliere et al., 2004), *in vivo* ischemia (Franke et al., 2004), epilepsy (Vianna et al., 2002), mechanical injury (Franke et al., 2001), transgenic models of Alzheimer's disease (AD) (Parathenani et al., 2003), as well as in human brain tissue samples from patients with sclerosis multiplex (Narcisse et al., 2005) and AD (Mclarnon et al., 2006).

The first study showing an up-regulation of the $P2X_7R$ in brain was that of Collo

et al. (1997) who found increased immunostaining for the $P2X_7R$ in activated microglia of the zona penumbra in the MCAO model. The microglial up-regulation of the $P2X_7R$ in response to oxygen deprivation has also been documented in retinal microglia (Morigiwa et al., 2000). In a more recent study, an early (1 day after MCAO occlusion) up-regulation of the $P2X_7R$ protein was observed in microglial cells, and later (4-7 days after MCAO occlusion) the receptor was also over-expressed in neurons and astrocytes of the pre-infarct area in spontaneously hypersensitive rats (Franke et al., 2004). $P2X_7R$ immunoreactivity appears to be up-regulated around amyloid plaques in activated microglia and astrocytes in Tg2576 transgenic AD mice having mutant amyloid precursor protein (APP) (Parvathenani et al., 2003). Expression of the $P2X_7R$ is also up-regulated in microglia obtained from AD individuals compared with levels in nondemented (ND) brain (McLarnon et al., 2006). In addition, the $P2X_7R$ was markedly up-regulated in amyloid-β $(A\beta)_{1\text{-}42}$-injected, compared with PBS- or reverse peptide $A\beta_{42\text{-}1}$-injected, rat hippocampus (McLarnon et al., 2006). $P2X_7R$ immunostaining has also been localized in reactive astrocytes around lesions in autopsy brain sections of multiple sclerosis patients (Narcisse et al., 2005).

Altogether these data indicate that the expression of the $P2X_7R$ is strongly activity-dependent during pathological conditions, suggesting that the receptor is a potential therapeutic target in neurodegenerative disorders. This has led to testing of protective roles of $P2X_7R$ antagonism in functional disease models. The neuroprotective action of non-selective P2 receptor antagonists is well documented in *in vitro* models of glutamate- (Volonte et al., 1999; Volonte and Merlo, 1996) and

kainate- (Zona et al., 2000) mediated excitotoxicity, glucose deprivation (Cavaliere et al., 2001b; Geng et al., 1997) and chemical hypoxia (Cavaliere et al., 2001a). At present, however, inhibition of the $P2X_7R$ as a strategy to protect neurons has not been well studied.

1.3. Glioma brain tumors

1.3.1. Glioma brain tumors: general

Gliomas are the most common brain tumors which originate from glial cells – astrocytes, oligodendrocytes, Schwann cells or their precursors. Astrocytomas are the most common, accounting for 4-5% of all cancer related deaths in North America (Kleihues and Caenee, 2000), and responsible for more than 40% of all central nervous system neoplasms (Kleihues et al., 1996). The World Health Organization (WHO) grades astrocytomas histopathologcially into four grades with increasing malignancy: Grade I and II astrocytomas can be considered as low-grade astrocytomas, while grade III and grade IV astrocytomas are highly malignant. Grade IV astrocytomas (glioblastoma multiforme; GBM) are composed of poorly differentiated cells with high mitotic activity and are specified by rapid growth and high vascularization accompanied by cell necrosis in less capillarized regions. Unfortunately, they are the most common primary brain tumors with mean survival time of only 5-12 months following diagnosis.

Traditional treatment options for malignant gliomas include surgery, radiation therapy, and chemotherapy. While therapies for high-grade gliomas are helpful, at

present, these treatments cannot cure these tumors. The two major reasons are that tumor cells infiltrate into surrounding brain and thus cannot be completely removed by surgery and that most glioma cells are at least partially resistant to radiation and chemotherapy. Therefore, high-grade glioma cells almost always start to grow again at some point in time.

Since these lethal brain tumors often outsmart traditional cancer treatment, new methods of therapy are required. In this regard a better understanding of malignant gliomas may help in the discovery of novel strategies to block their development and progression. In our study, rat C6 glioma has been used as an experimental model system.

1.3.2. C6 glioma: the experimental model system

The C6 glioma cell line is a widely used cell line in neurobiological research and has been used as an experimental model system to elucidate the mechanisms underlying the aggressive nature of glioma brain tumors. Compared to primary neural cells, the C6 cell line offers some major advantages, such as easy accessibility and culture as well as availability of high cell numbers.

The rapidly proliferating rat C6 glioma cell line was originally induced in random-bred Wistar-Furth rats by exposure to *N,N'*-nitroso-methylurea (Benda et al. 1968). When transplanted into rat brains, C6 cells form tumors with many characteristics of malignant glioma and the tumor formed by C6 cells was histopathologically classified as astrocytoma (Grobben et al., 2002).

The C6 glioma has been and will continue to be useful for a variety of studies related to brain tumor biology including studies on tumor growth, invasion and migration, capillary permeability, blood-brain barrier disruption, neovascularization, growth factor regulation and production and biomedical studies. It has also been used to evaluate the therapeutic efficacy of cancer treatments such as chemo-, radiation-, immuno- and gene therapies (Barth, 1998).

In the C6 glioma model, a number of factors have been shown to participate in tumor growth, invasion and angiogenesis (extensively reviewed by Grobben et al., 2002). In addition, recent evidence suggests that ATP signaling may also be involved in glioma development and that P2 receptors might provide novel therapeutic targets in the treatment of brain cancer (Morrone et al., 2005; Morrone et al., 2006). Several purinergic receptors of the P2YR family are expressed in C6 cells including $P2Y_1$, $P2Y_2$ and $P2Y_{12}$ (Czajkowski et al., 2002; Sabala et al., 2001). P2YR have been reported to mediate changes in $[Ca^{2+}]i$ following ATP application (Sabala et al, 2001) and to regulate glioma cell proliferation (Czajkowski et al., 2004). 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.

1.3.3. Microglia in brain tumors

As noted above, microglia play important roles in inflammatory diseases of the CNS. These cells have been identified in brain neoplasms as well. The presence of

microglia in brain tumors was first reported by Rio-Hortega and Asua in 1921 (Rio-Hortega and Asua, 1921) and Penfield in 1925 (Penfield, 1925). In the 1980s, expanding interest in immunotherapy of brain tumors prompted the use of immunohistochemistry to characterize the inflammatory cell response to brain tumors (Rossi et al., 1988, 1987, 1989). These initial findings were confirmed later in both human and animal brain tumors, where a significant macrophage and microglia infiltration was demonstrated (Roggendorf et al., 1996; Shinonaga et al., 1988; Streit, 1994; Wierzba-Bobrowicz et al., 1994). Some studies also suggested a direct correlation between the grade of gliomas and levels of resident tumor microglia/macrophage (MG/MP) cells, indicating that gliomas may play an active role in MG/MP recruitment (Roggendorf et al., 1996).

An interesting question is if the MG/MP response is due to local tissue damage or active chemoattraction by tumor cells. However, it has been known that a variety of chemokines and growth factors can be released directly from tumor cells themselves or as a result of local tissue injury due to tumor growth. This can result in recruitment of microglia and macrophages from two main sources: resident brain microglia or perivascular macrophages that can become activated and migrate toward brain tumors (Figure 1-2). The specific cytokins/chemokines involved in glioma recruitment of microglia remain poorly understood. However, MCP-1 is believed to be a major contributor to MG/MP recruitment to gliomas. MCP-1 is expressed at both mRNA and protein levels in gliomas (Leung et al., 1997) and it has been shown to functionally recruit microglia to gliomas *in vivo*, while promoting their proliferation (Platten et al., 2003).

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Figure 1-2. Possible interactions between neoplastic cells, microglia, and macrophages in brain tumors (Watters et al., 2005)

At present, the biological role of microglia and macrophages in gliomas remains unknown. It is also not known if the presence of microglia in and around tumors is an attempt by the immune response to combat the tumor or if microglia are recruited by the tumor to promote tumor growth and proliferation.

It has become increasingly clear that the defense functions of immune cells such as lymphocytes and microglia are compromised and the well-known immunosuppressive, intratumoral milieu of gliomas is partially due to the action of cytokines and important regulatory proteins (Schneider et al., 1992). Gliomas produce several immunsuppressive cytokines and factors to inhibit cytotoxic T-cell activation as well as microglial activation. All interleukin (IL)-6 type (strongly immunosuppressive) cytokines IL-1, IL-11, ciliary neurotropic factor (CNTF), cardiotrophin (CT)-1, leukemia inhibitory factor (LIF) and oncostatin-M (OSM)) have been demonstrated at the protein and mRNA levels in glioma cell lines (Goswami et al., 1998; Halfter et al., 1998a, b; Hao et al., 2002; Murphy et al., 1995), as have all tumor growth factor (TGF)-β cytokine family members (Constam et al., 1992; Hao et al., 2002; Olofsson et al., 1992). TGF-β inhibits the proliferation of microglia as well as their production of cytokines *in vitro* (Suzumura et al., 1993), whereas IL-6 and IL-10 have been postulated to promote glioma cell line proliferation (Goswami et al., 1998; Huettner et al., 1997). Glioma cells secrete IL-6 (Goswami et al., 1998) and infiltrating MG/MP are the main source of IL-10 (Huettner et al., 1997; Wagner et al., 1999). The immunosuppressive cytokine IL-10 not only promotes glioma cell proliferation but also enhances their ability to migrate *in vitro* (Huettner et al., 1997). Prostaglandin E_2 (PGE_2) is another important immunosuppressant in

gliomas. Cyclooxygenase-2 (COX-2) is one of the major enzymes responsible for the arachidonic acid conversion to prostaglandins and it has been demonstrated that in the C6 tumor model, glioma-infiltrating microglia, not the glioma cells themselves, are the major source of COX-2 activity (Badie et al., 2003).

On the other hand, the presence of MG/MP in and around gliomas suggests that they may play a functional role in glioma survival. Many substances produced by microglia may contribute to glioma proliferation, migration and angiogenesis.

The proliferation factor epidermal growth factor (EGF) can be produced by activated microglia *in vitro* (Briers et al., 1994) and microglia express receptors for EGF that enable them to proliferate in response to local release of this growth factor (Nolte et al., 1997). Both microglia and gliomas secrete vascular endothelial growth factor (VEGF) (Lafuente et al., 1999; Tsai et al., 1995), known to be a critical angiogenic factor. *In vitro*, VEGF can also induce proliferation and migration of microglia (Forstreuter et al., 2002). VEGF production may thus facilitate glioma survival and promote the recruitment and proliferation of microglia whose production of tumor survival factors can also contribute to glioma growth and malignancy. Substance P (SP) and its receptor NK-1 are expressed in microglia (Lai et al., 2000; Rasley et al., 2002) and in human gliomas (Palma and Maggi, 2000; Palma et al., 1999). SP induces microglial release of cytokines involved in angiogenesis and tumor cell proliferation, including IL-1β and TNF-α (Luber-Narod et al., 1994; Martin et al., 1993). IL-1β has been shown to be involved in the invasiveness and angiogenesis of several tumor types *in vivo* (Saijo et al., 2002; Sunderkotter et al., 1994; Voronov et al., 2003), and it increases the expression and activity of Matrix metalloprotease-9

(MMP-9) *in vitro* (Esteve et al., 1998, 2002; Nakano et al., 1995), which is thought to be critical for local invasion of brain tissue by tumor cells. Microglia also highly express MMP-9 *in vitro* in response to chemokines such as MCP-1 (Cross and Woodroofe, 1999). Therefore, microglia may be attracted to gliomas and stimulated to produce factors permitting glioma invasion and metastases.

TNF-α is produced by microglia and can have very potent effects on glioma biology. According to previous studies, it is likely that the major source of this factor within gliomas is microglial cells (Hao et al., 2002). TNF-α induces the migration of leukocytes into gliomas (Tada et al., 1993) while increasing the expression of factors involved in tumor cell migration and angiogenesis in glioma cells. The factors include VEGF (Ryuto et al., 1996), EGF receptor (Adachi et al., 1992), and MMP-9 (Esteve et al., 2002).

1.4. Research hypotheses

1) I postulate that $P2X_7$ receptors are functionally expressed in C6 glioma cells. Furthermore, activation of $P2X_7R$ is predicted to mediate changes in intracellular calcium levels and are coupled to cell functional responses.
2) Pharmacological modulations of the $P2X_7R$ will alter tumor development *in vivo*.

In this study, calcium imaging and molecular biology methods have been applied to examine $P2X_7R$-mediated responses *in vitro* in cultured C6 cells and the rat C6 glioma model has been used to investigate the properties of the $P2X_7R$ *in vivo*

2. Materials and Methods

2.1. In vitro studies

2.1.1. Cell Culture

Glioma C6 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells from passage number 39 to 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 1.5g/L sodium bicarbonate (Javitt et al., 2005) 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.

2.1.2. Calcium-Sensitive Spectrofluorescence

The methods for preparation of cells for calcium imaging followed published procedures (McLarnon et al., 2005). 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 hr; 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-sec 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.

2.1.3. 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'-GCTCTCTTGGGTGCACTGGA-3' and rat VEGF antisense 5'-CACCGCCTTGGCTTGTCACA-3' (644 bp); rat β–actin sense 5-GTGGGGCGCCCCAGGCACCA-3' and rat β–actin antisense 5'-GTCCTTAATGTCACGCACGATTTC-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 sec, 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 ImageJ 1.37b software (National Institute of Health, Bethesda, MD) and expressed as relative mRNA levels (mRNA levels normalized to β-actin).

2.1.4. Western blot analysis

Total proteins from the C6 glioma cell line or rat brain tissues were prepared as described previously (Hu et al., 2003). Protein samples were analyzed by SDS-PAGE and Western blot analysis (Ryu et al., 2006). 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, Amersham-Pharmacia Biotech). The signals were captured by Fluor-S MultiImager and analyzed using Quantity One software (Bio-Rad).

2.1.5. Immunocytochemistry of C6 glioma cells

For immunocytochemical staining, C6 glioma cells grown on poly-L-lysine-coated Aclar plastic coverslips were incubated in the standard medium with BzATP (300 μM; Sigma) in the presence or absence of OxATP (300 μM) for 8 hr. In some experiments, cells were pre-treated with OxATP for 2 hr. After incubation, cells were rinsed in 0.1 M PBS and fixed in 4% paraformaldehyde for 10 min and placed in PBS containing 1% bovine serum albumin, 10% normal goat serum and 0.2% Triton X-100 for 30 min. The cells were then incubated with rabbit anti-$P2X_7R$ antibody (1:500, LifeSpan Biosciences, Seattle, WA) for 48 hr at 4°C, followed by biotinylated anti-rabbit IgG (1:1000; Vector, Burlingame, CA) for 1 hr and avidin-biotin complex (1:1000; Vector) for 1 hr at room temperature. The reaction product was visualized with 0.05% 3,3′-diaminobenzidine (Sigma) and 0.003% H_2O_2. After a PBS wash, coverslips were mounted onto glass slides using gelvatol and examined under a microscope. For negative staining controls, primary antibody was omitted during the staining procedure. For quantitative analysis, four fields in each coverslip were selected (magnification of 40X) and images were digitized using a Zeiss Axioplan-2 light microscope equipped with a DVC camera (Diagnostic Instruments, Sterling Heights, MI). Digitized images were then analyzed using Northern Eclipse software (Empix Imaging). The staining was quantified by measurement of the pixel intensity

above a predetermined threshold level of staining intensity. Quantification was done in a blinded manner.

2.1.6. Ethidium bromide (EtBr) uptake assay

C6 cells were seeded in 6-well plates and were allowed to grow to confluency in standard culture media. In some experiments, cells were treated with 300 μM OxATP for 2 hr at 37°C prior to performing the EtBr uptake assay. Cells were then centrifuged, collected and suspended with PBS at 1×10^6 cells/ml in 1.5 ml Eppendorf tubes and then incubated for 5 min at room temeperture with EtBr (25 μM), with or without treatment (BzATP alone, BzATP plus OxATP or OxATP alone). After incubation, cells were centrifuged and supernatant was discarded prior to adding distilled water to rupture plasma membrane. An Ultrospec 2100 *pro* UV/Visible spectrophotometer (GE Healthcare, Uppsala, Sweden) was used to measure the absorbance of the samples at 488 nm (the excitation wavelength of EtBr). The reference cuvette contained the lysate from cells that were not incubated with EtBr or any other compound. Four independent experiments were performed and the results were normalized relative to the untreated control and presented as mean percentage. Quantification was done in a blinded manner.

2.1.7. Scratch-wound migration assay

The methods for the scratch-wound migration assay followed published procedures (Faber-Elman et al., 1996; Lind et al., 2006) with some modifications. C6 cells were plated onto coverslips and were allowed to grow to confluency in the same

medium as for cell culture. Once cells were confluent, the medium was switched to serum-free F12K for 24 hr to minimize the influence of growth factors. After the period of serum starvation, the confluent monolayers were scratched using a 200 μl sterile plastic pipette tip. In some experiments, cells were incubated with 300 μM OxATP for 2 hr or 1μM BBG (Sigma) for 15 min prior to performing the scratch wound. The coverslips were then washed three times with the serum-free F12K, and treatment was applied. BzATP was added alone or with OxATP (or BBG) in the serum-free medium. After 24 hr of incubation (37°C, 5% CO_2), the cells were fixed for DAPI staining. Cells were washed in PBS twice and then fixed with 4% paraformaldehyde in 0.1 M PBS (10 min at room temperature). After wash in PBS, cells were incubated in DAPI (Invitrogen) at 1 μg/ml in PBS to visualize nuclei and determine cell numbers in the field of view. Cells were then washed in water and mounted onto glass slides using gelvatol. The extent of the repopulation of the wound area by DAPI-stained cells was assessed in digitalized images, using the NIH Image J 1.37b software (National Institute of Health). For quantification of data, overall areas of the wound were first examined. Consistent patterns of uniform migration throughout the experimental region were observed. We then quantified the number of cells in a representative area of 0.5×0.5 mm^2. Four experiments were performed in triplicate. The results were normalized relative to the number of cells counted in equal areas in the control untreated cultures and are presented as mean percentage. Quantification was done in a blinded manner.

2.1.8. Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Statistical significance ($p < 0.05$) was evaluated using Student *t*-test or one-way analysis of variance followed by Student-Newman-Keuls multiple comparison test when applicable (GraphPad Prism 3.0; San Diego, CA).

2.2. In vivo studies

2.2.1. Intracerebral implantation of C6 cells

All animal procedures were carried out according to protocol approved by the UBC Animal Care Ethics Committee, adhering to guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (250-300 g; Charles River Laboratories, St. Constant, PQ) were anesthetized with intraperitoneal (i.p.) injection of ketamine hydrochloride (72 mg/kg; Bimeda-MTC, Cambridge, ON) and xylazine hydrochloride (9 mg/kg; Bayer Inc., Etobicoke, ON) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). For implantation of glioma cells, C6 cells were harvested, centrifuged and suspended in HEPES-buffered physiological salt solution (HPSS) and 3 µl of cell suspension (1×10^5 cells/µl) was injected into the striatum of each animal. Injection coordinates for the striatum were as follows: anteriorposterior (AP): 1.0 mm; mediallateral (ML): -3.0 mm; dorsoventral (DV): -5.0 mm, from bregma. A midline skin incision was made in the scalp to expose the skull and stereotaxic unilateral transplantation of C6 glioma cells was performed as previously described (Ryu et al., 2004). In some experiments, C6/LacZ cells (ATCC,

Manassas, VA) were used. They are modified C6 cells that constitutively express the lacZ reporter gene product, E. coli-derived beta galactosidase (β-gal). The cells were maintained under the same conditions as C6 glioma cells (see **2.1.1.**). C6 cells were slowly injected (0.2 μl/min) into the striatum using a 10 μl Hamilton syringe attached to a 26-gauge needle. The injection syringe was left in place for an additional 3 min and the needle was slowly withdrawn. After removing the needle, the skin was sutured and the animals were allowed to recover and then returned to their cages.

2.2.2. Immunohistochemical analysis

Animals were transcardially perfused with heparinized cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (0.1 M PB, pH 7.4) under ketamine/xylazine anesthesia. Brains were removed from the skull, post-fixed in the same fixative overnight and then placed in 30% sucrose for cryoprotection. The brains were then rapidly frozen in powdered dry ice and stored at -70°C. Coronal sections (40 μm) were cut on a cryostat at the interval of 0.8mm throughout the striatum and hippocampus and stored in cryoprotectant solution. Free-floating sections were processed for single immunohistochemistry, as described previously (Ryu and McLarnon, 2006). Briefly, sections were permeabilized by incubation in PBS containing 1% bovine serum albumin (BSA; Sigma), 10% normal goat serum (NGS) and 0.2% Triton X-100 for 1 hr. Sections were then incubated overnight at 4°C with the following primary antibodies: anti-CR3R (OX-42, a marker for microglia/macrophages, 1:500; Serotec, Oxford, UK), anti-glial fibrillary acidic

protein (GFAP, a marker for astrocytes, 1:1000; Sigma), or anti-neuronal nuclei (NeuN, a marker for neurons, 1:1000; Chemicon, Temecula, CA). Sections were rinsed in PBS with 0.5% BSA and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200; Molecular Probes) or Alexa Fluor 594-conjugated goat anti-mouse IgG (1:200; Molecular Probes) at room temperature for 2 hr in the dark. Sections were then washed in 0.1M PBS, mounted on Superfrost/Plus microscope slides (Fisher Scientific; Pittsburgh, PA), and mounted in DPX Mountant (Fluka, Toronto, ON, Canada). For the negative control, the primary antibody was omitted from the staining procedures.

2.2.3. Double immunofluorescence staining

Double immunofluorescence staining was performed as described previously (Ryu and McLarnon, 2006). Free-floating sections were incubated overnight at 4°C with a mixture of two primary antibodies: anti-$P2X_7R$ (1:200; Alomone Labs, Jerusalem, Israel) in combination with either anti-β-gal (1:200; Abcam, Cambridge, MA), anti-OX-42 (1:500; Serotec), anti-GFAP (1:1000; Sigma) or anti-NeuN (1:500; Chemicon). Sections were then incubated in a mixture of Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:100; Molecular Probes, Eugene, OR) and Alexa Fluor 594-conjugated goat anti-mouse IgG (1:100; Molecular Probes) at room temperature for 2 hr in the dark

2.2.4. BBG administration

In some experiments, drug administration was started right after C6 implantation. Conscious rats (n = 3) were injected i.p. with BBG (50 mg/kg, dissolved in PBS) twice per day and control animals (n = 3) received an equivalent volume of pyrogen-free saline. Two weeks after tumor transplantation, all animals were sacrificed and coronal sections were taken as described in **2.2.2.** Nissl staining was then performed to determine tumor volumes.

2.2.5. Quantification of tumor volumes

Tumor volumes were determined from coronal striatal sections (40 μm at the interval of 0.8 mm) stained for Nissl substance. First, we measured the absolute tumor area using the computer program NIH ImageJ 1.37b (National Institute of Health). The absolute tumor volume was then calculated after integration of the area measurements from every section and the relative tumor volume was expressed as the percentage of the tumor volume to the volume of the intact hemisphere.

3. Results

3.1 In vitro studies

3.1.1. Intracellular calcium levels are altered in response to $P2X_7R$ agonist BzATP

Initial experiments examined effects of the specific $P2X_7$ agonist, BzATP, to increase mobilization of $[Ca^{2+}]i$ in C6 glioma cells. A representative response elicited by BzATP (300 μM) is presented in Figure 3-1A and shows the ligand induces a rapid rise in $[Ca^{2+}]i$ (measured as ratio F340/380) followed by a slowly decreasing component (mean response from n = 38 cells). The $[Ca^{2+}]i$ increase induced by BzATP was largely inhibited in the presence of the $P2X_7R$ antagonist OxATP (300 μM) (Figure 3-1B; n = 30 cells). Quantification for the amplitudes of responses for BzATP, in the absence and presence of OxATP, is presented in Figure 3-1C; responses were diminished by 67% with application of OxATP (N = 5 experiments with a total of 132 cells for BzATP treatment and 151 cells for BzATP treatment in the presence of OxATP).

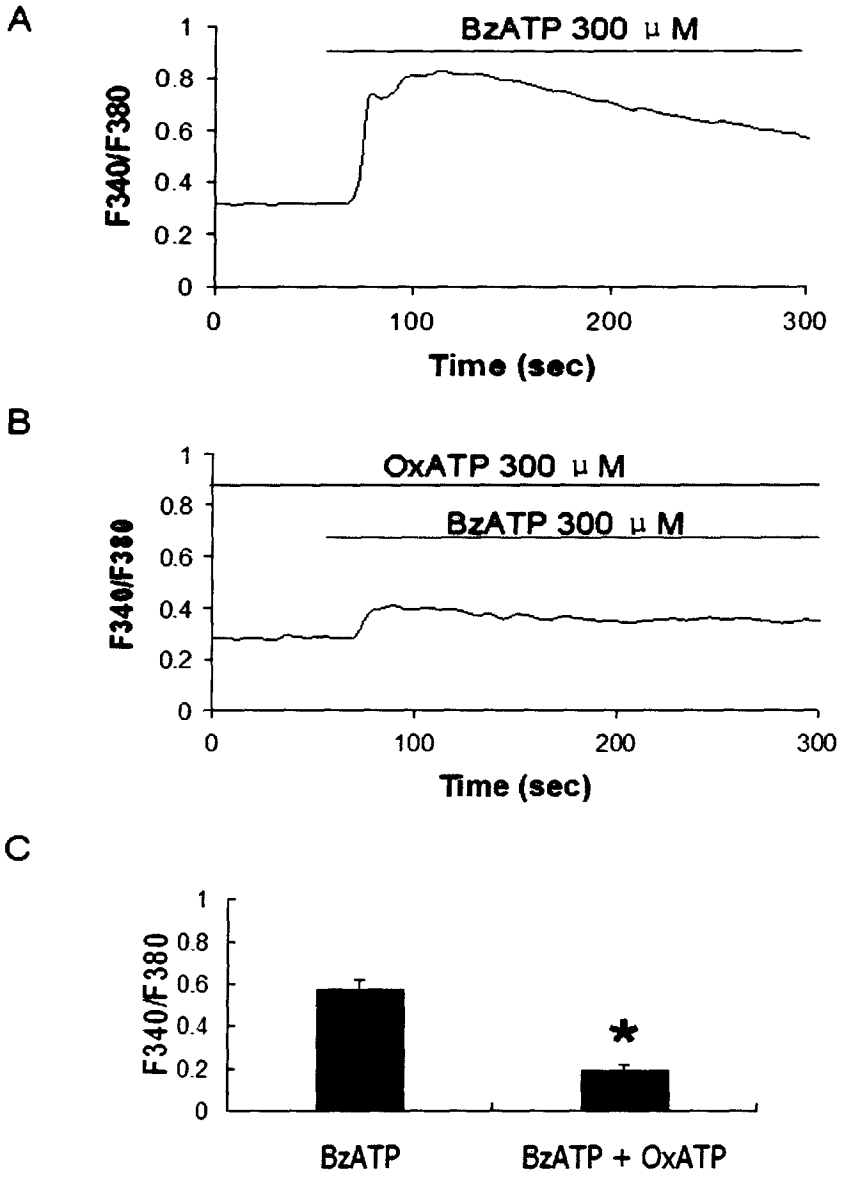


Figure 3-1. $[Ca^{2+}]i$ responses induced by the $P2X_7$ agonist BzATP
Representative traces show Ca^{2+} responses induced by BzATP alone (Fig. 1(A); n = 38 cells) or (B) in the presence of OxATP added 2 hr before BzATP application (n = 30 cells). (C) Amplitudes of responses for different treatments are shown (N = 5 independent experiments for each group). *$p < 0.05$.

3.1.2. $P2X_7$ receptors are expressed in C6 cells and regulate expression of pro-inflammatory factors

Expression of $P2X_7R$ and several inflammatory cytokines was examined using RT-PCR with C6 cells exposed to control (standard medium alone) or 300 μM BzATP solution (treatments for 8 hr). Representative results are presented in Figure 3-2A and show in control low levels of basal expression of $P2X_7R$, the chemokines MCP-1 and IL-8, and the angiogenic factor VEGF. Treatment with BzATP caused marked increases in expression of $P2X_7R$ and levels of the inflammatory factors (Figure 3-2A). Semi-quantitative results (Figure 3-2B; from N = 4 independent experiments) showed that stimulation with the $P2X_7R$ ligand significantly increased expression of $P2X_7R$ and all three factors (relative to control) as follows: $P2X_7R$ (by 116%), MCP-1 (by 549%), IL-8 (by 140%), and VEGF (by 62%). Surprisingly, OxATP was ineffective in blocking the BzATP-induced increase in expression of the pro-inflammatory factors (data not shown); this point is discussed below.

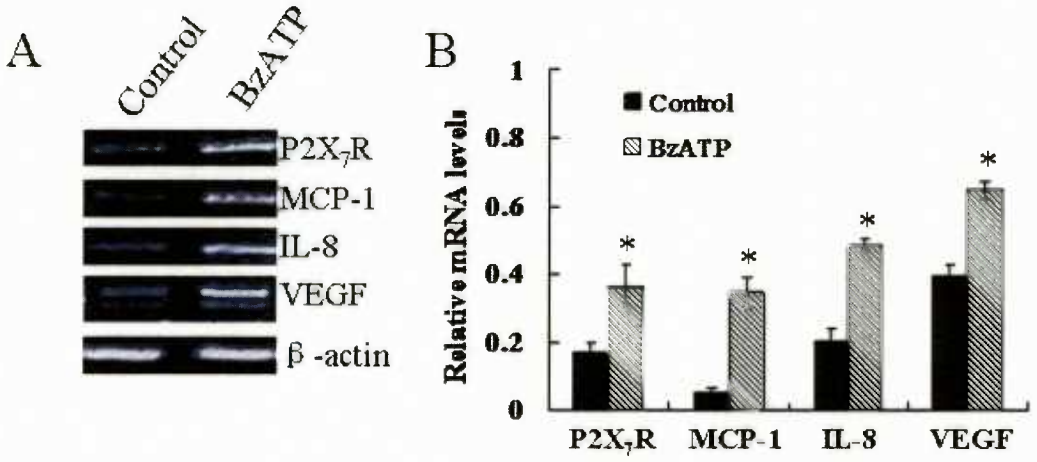


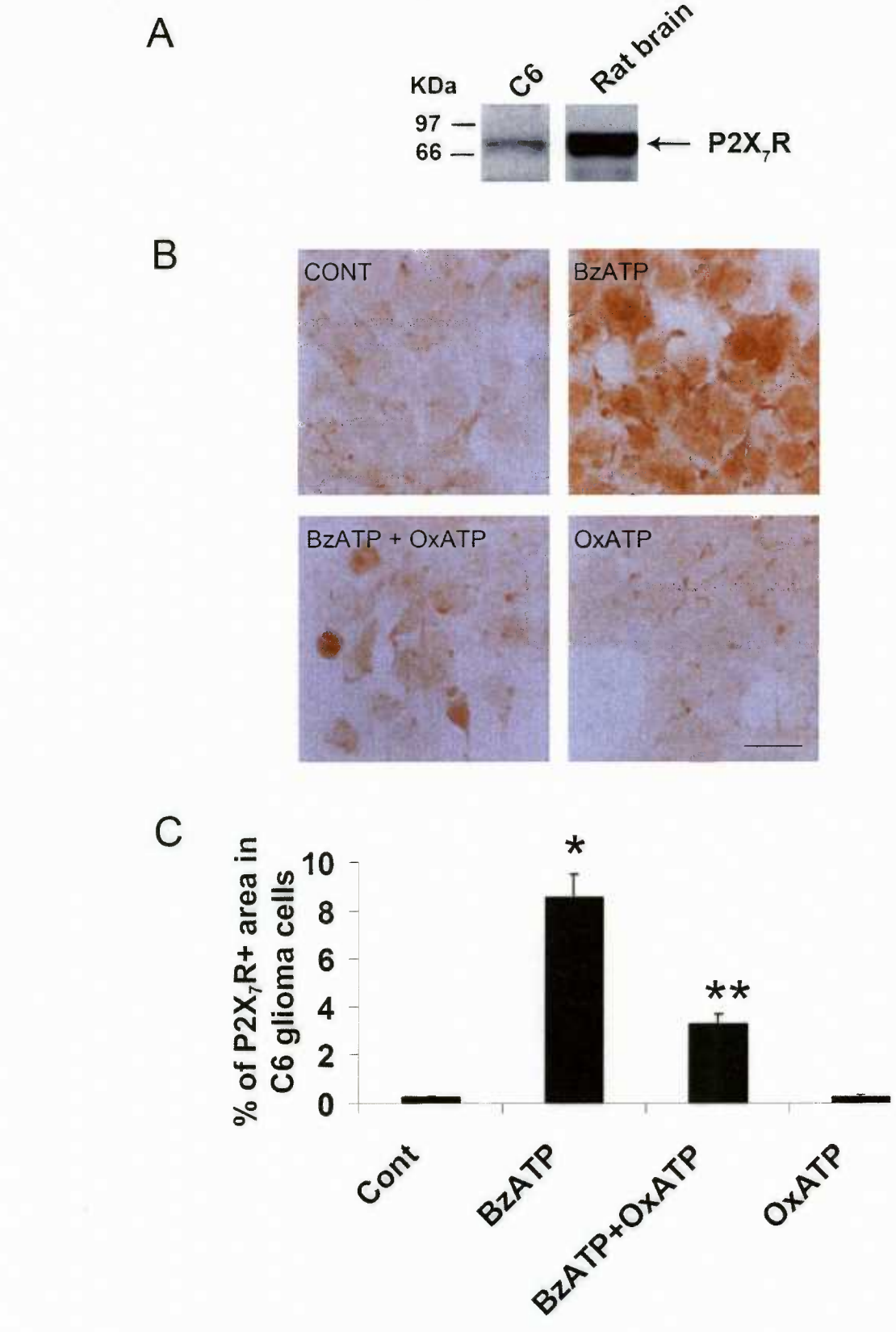
Figure 3-2. Effects of BzATP stimulation of C6 glioma cells on expression of $P2X_7R$ and pro-inflammatory factors

(A) Representative RT-PCR results for $P2X_7R$, MCP-1, IL-8 and VEGF for exposure of C6 to control (standard medium) and BzATP (300 μM for 8 hr). β-actin served as a reaction standard. (B) Overall results from N = 4 independent experiments. *$p < 0.05$.

3.1.3. $P2X_7R$ protein is present in C6 cells and is up-regulated by BzATP stimulation

Western blotting and immunocytochemical staining were used to determine whether the $P2X_7R$ protein is produced by C6 cells and if production of the $P2X_7R$ is modulated with cell exposure to BzATP. Western blot analysis using antibodies against $P2X_7R$ demonstrated the presence of $P2X_7R$ protein in C6 cells (Figure 3-3A) and cell lysates from rat brain tissues were used as positive controls. This result was further corroborated by immunocytochemical staining. Representative results show that under unstimulated conditions, immunoreactivity for $P2X_7R$ was low (Figure 3-3B, upper left panel). BzATP markedly enhanced $P2X_7R$ protein levels (Figure 3-3B upper right panel) which were reduced if OxATP was included with BzATP (Figure 3-3B lower left panel). OxATP alone did not alter immunoreactivity for $P2X_7R$ protein levels (Figure 3-3B lower right panel). Quantification of the results (from N = 3 independent experiments) is presented in Figure 3-3C and shows significant increases in $P2X_7R$ protein levels with BzATP (by 34-fold relative to the untreated control) and marked inhibition in the presence of OxATP (by 61% relative to BzATP alone).

Figure 3-3. BzATP-induced up-regulation of $P2X_7R$ protein in C6 glioma
(A) Expression of $P2X_7R$ protein in C6 cells was analyzed by Western blotting in cell lysates using specific $P2X_7R$ antibodies. Lysates from rat brain tissues were used as positive controls. (B) Representative immunocytochemical staining for control (upper left panel), BzATP (upper right panel), BzATP+OxATP (lower left panel) and OxATP alone (lower right panel); Scale bar = 10 μm. (C) $P2X_7R$ protein expression in C6 cells for the different treatments (N = 3 independent experiments); * $p<0.05$ for BzATP vs control and ** for BzATP+OxATP vs BzATP alone.


A
KDa
C6
Rat brain
97
66
P2X7R
B
CONT
BzATP
BzATP + OxATP
OxATP
C
% of P2X7R+ area in C6 glioma cells
10
8
6
4
2
0
*
**
Cont
BzATP
BzATP+OxATP
OxATP

3.1.4. $P2X_7R$ activation induces pore formation in C6 cells

An ethidium bromide (EtBr) uptake assay was used to examine $P2X_7R$-induced pore formation. The overall results (Figure 3-4, N = 4 for each group) show incubation with BzATP (300 μM, 5 min) increased absorbance (measured at 488 nm) by more than two-fold compared with untreated controls indicating pore-formation was induced with $P2X_7R$ activation. Treatment of OxATP with BzATP significantly inhibited dye uptake (by 58%) compared to BzATP application alone. OxATP applied separately did not alter absorbance relative to control. Interestingly, although $P2X_7R$ activation leads to formation of large pores, exposure of C6 cells to a maintained stimulation of a high level of BzATP (at 500 μM for 72 hr) had no effect to diminish cellular viability (data not shown). Thus, chronic stimulation of $P2X_7R$ was not linked to activation of apoptosis or necrosis pathways in C6 cells.

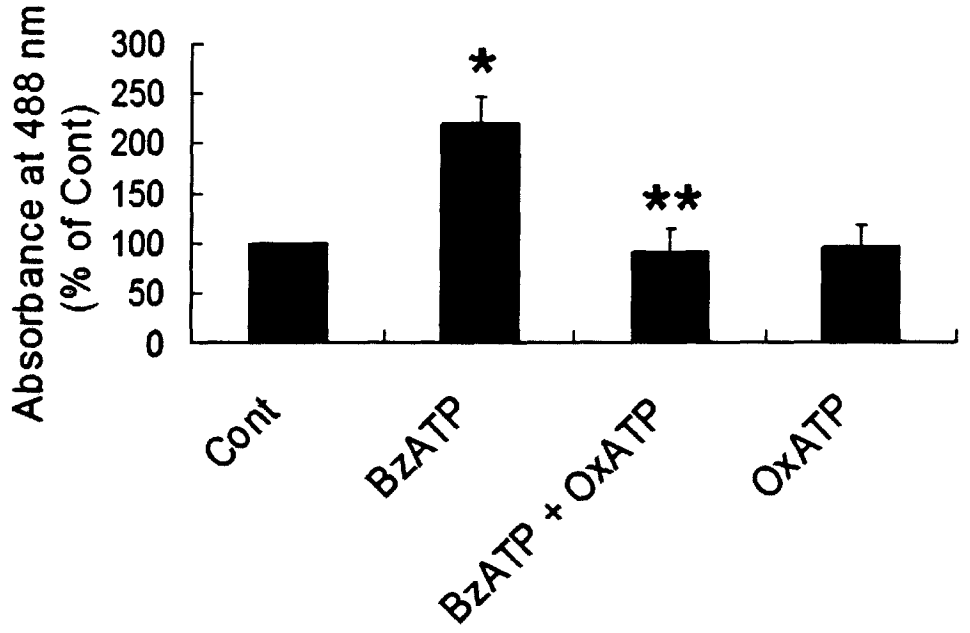


Figure 3-4. BzATP-induced uptake of ethidium bromide
N = 4 independent experiments for each treatment; * $p<0.05$ for BzATP vs control and ** for BzATP+OxATP vs BzATP alone.

3.1.5. $P2X_7$ receptors in C6 glioma cells regulate cell mobility

The mobility of C6 glioma was also investigated in this work using a scratch-wound assay. As shown in Figure 3-5A (top left panel), little or no migration of C6 was observed in control serum-free condition. However, in the presence of BzATP (300μM, 24 h incubation) C6 cells showed considerable mobility (Figure 3-5A, top right panel) which was largely inhibited in the presence of OxATP (300μM; lower left panel). No mobility of C6 was observed with OxATP alone (Figure 3-5A, lower right panel). Quantification of the results (from N = 4 independent experiments) is presented in Figure 3-5B and shows a significant increase in C6 mobility with exposure to BzATP (by 290% relative to the untreated control) and marked inhibition in the presence of OxATP (by 91% relative to BzATP alone). Interestingly, levels of C6 mobility were higher in control compared with OxATP suggesting that basal expressions of $P2X_7R$ were sufficient to permit C6 mobility in unstimulated cells. Another $P2X_7R$ agonist, BBG, showed similar effects in blocking cell mobility induced by BzATP (Figure 3-5C; by 74% relative to BzATP alone) while treatment with BBG alone did not affect C6 cell migration.

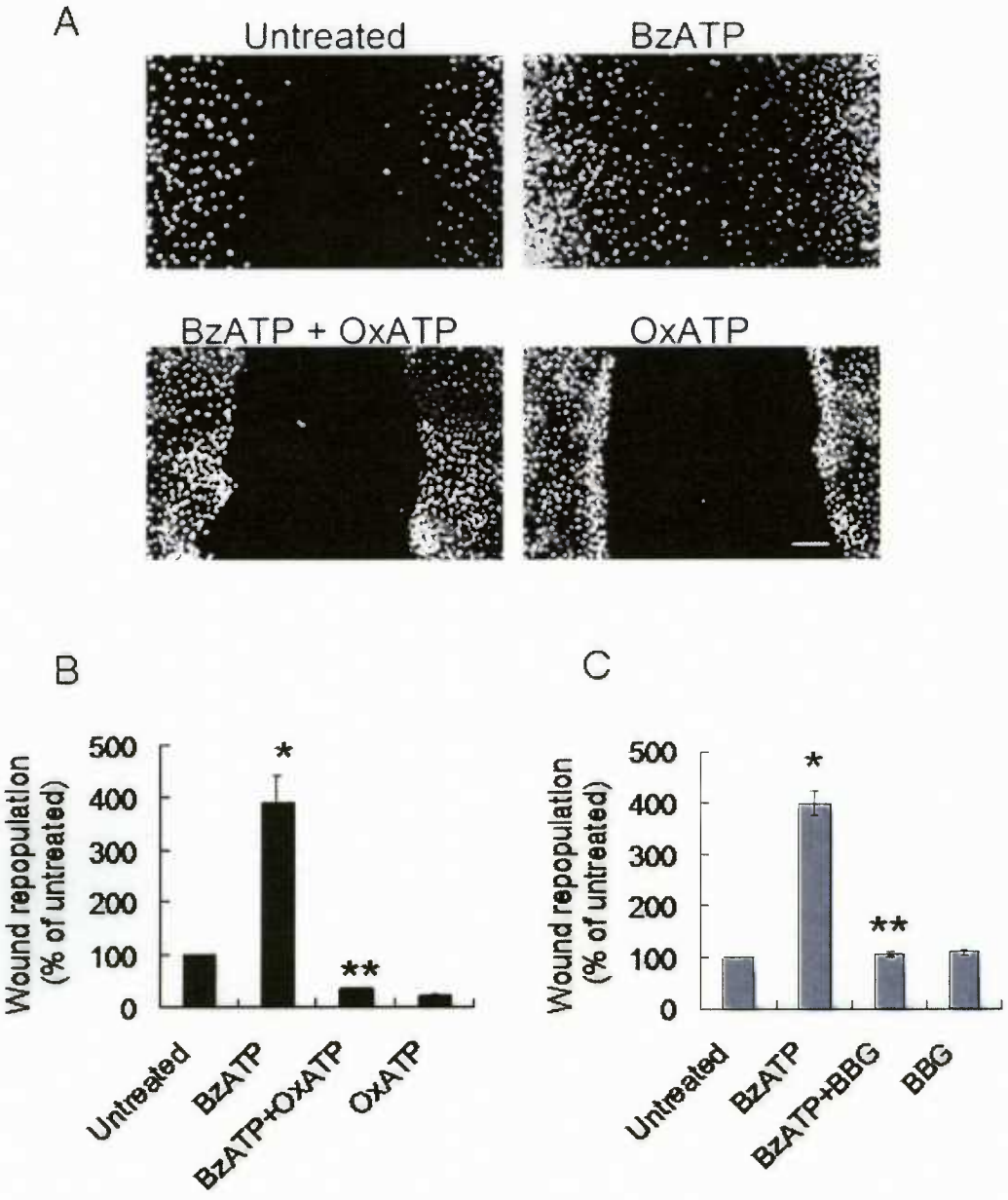


Figure 3-5. BzATP effects on C6 repopulation of the wound area

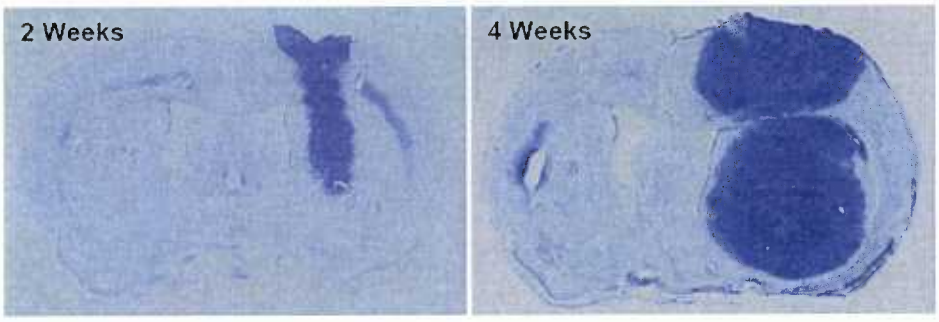
(A) Representative results for treatments with control (upper left panel), BzATP (upper right panel), BzATP+OxATP (lower left panel) and OxATP alone (lower right panel). Scale bar = 100 μm. Experiments with BBG showed similar results. (B) Quantification for wound repopulation with OxATP as the $P2X_7R$ antagonist from four independent experiments performed in triplicate. (C) Quantification for wound repopulation with BBG as the $P2X_7R$ antagonist from four independent experiments performed in triplicate; * $p<0.05$ for BzATP vs untreated control and ** for BzATP+OxATP or BzATP+BBG vs BzATP alone.

3.2. In vivo studies

3.2.1. Tumor progression in the rat brain

Rodent C6 tumor model was used to investigate $P2X_7R$ expression and function *in vivo*. Initial experiments used immunohistochemical methods to determine tumor progression at two and four weeks following C6 injection into rat striatum. As shown in Figure 3-6A, Nissl staining indicates tumor development at two weeks (2 W) and four weeks (4 W) after transplantation. Quantification (from n = 3 animals per group) is provided in Figure 3-6B and the relative tumor area is expressed as the percentage of the area occupied by the tumor to the area of the contralateral side.

A



B

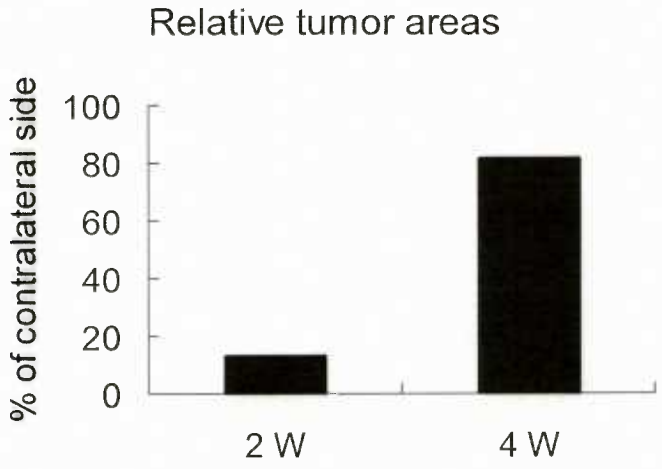


Figure 3-6. Progression of C6 glioma

(A) Nissl staining shows development of tumor at 2 and 4 weeks after C6 transplantation. No Nissl staining of C6 glioma was evident in the contralateral side. (B) Quantification of tumor areas (n = 3 animals per group).

3.2.2. Co-localization of $P2X_7R$ with C6/LacZ cells

In order to label injected C6 cells *in situ*, we used C6/LacZ cells. The cells constitutively express the lacZ reporter gene product, E. coli-derived beta galactosidase (β-gal), as revealed on tissue sections by histochemical stain, and single tumor cells can be identified. At 2W after injection, expression of $P2X_7R$ associated with C6 cells was examined. Tumor development at 2W following C6/LacZ injection was shown by Nissl staining (left panel, Figure 3-7A) and single C6 cells were identified by β-gal immunoreactivity (ir) (right panel Figure 3-7A). A high degree of co-localization of $P2X_7R$ and C6 was observed (top panel, Figure 3-7B), indicating that C6 cells express $P2X_7R$ *in situ*. Intratumoral regions show marked β-gal and $P2X_7R$ staining (bottom left panel, Figure 3-7B) while boundary regions show β-gal ir in intratumoral, but not peritumoral regions (bottom right, Figure 3-7B).

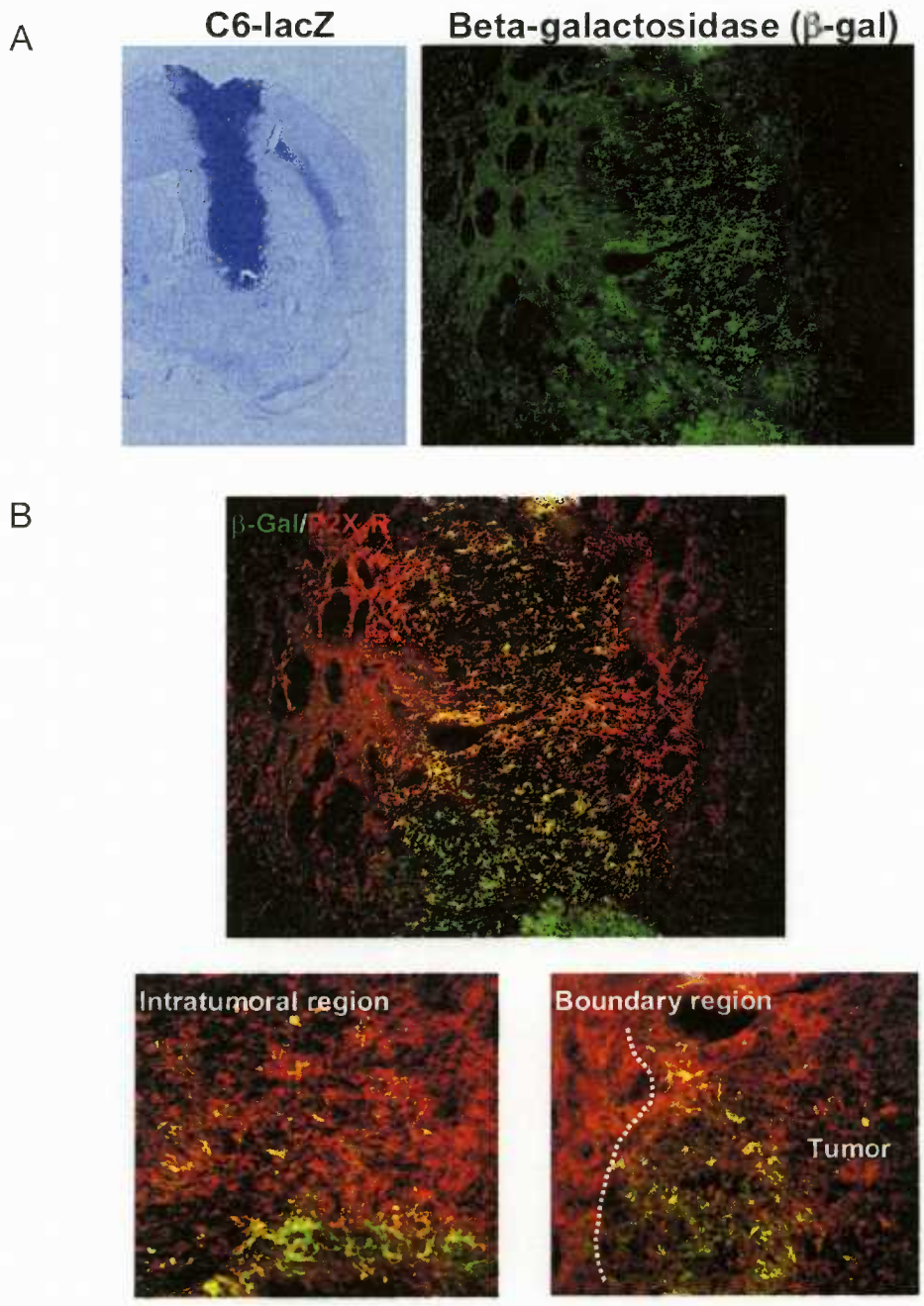


Figure 3-7. Co-localization of $P2X_7R$ with C6/LacZ cells

(A) Nissl staining shows development of C6 glioma at 2 weeks after C6/LacZ transplantation (left panel) and C6 cells were also identified by β-gal ir (right panel). (B) Co-localization of $P2X_7R$ (red) with C6/LacZ cells (green).

3.2.3. Co-localization of $P2X_7R$ with microglia/macrophages and astrocytes

We then examined expression of $P2X_7R$ associated with microglia/macrophages (Figure 3-8) and astrocytes (Figure 3-9) (microglia/macrophages; OX-42 marker; astrocytes; GFAP marker). Considerable OX-42 and $P2X_7R$ ir was observed in striatum (top panel, Figure 3-8) with little ir evident in contralateral regions (second panel, Figure 3-8). Intratumoral areas show high levels of staining for OX-42 and $P2X_7R$ (third panel, Figure 3-8). Boundary regions show co-localization of $P2X_7R$ and OX-42 in intratumoral, but not peritumoral ($P2X_7R$ ir in peritumoral region is likely astrocytic).

Astrocyte and $P2X_7R$ immunostaining is presented in Figure 3-9 (top panel) with only low levels of ir observed in contralateral striatum (second panel, Figure 3-9). GFAP ir was minimal in intratumoral regions (third panel, Figure 3-9). Co-localization of GFAP positive astrocytes with $P2X_7R$ was evident in the peritumoral portion of the boundary region (bottom panel, Figure 3-9). No $P2X_7R$ ir was observed with neurons (NeuN marker, data not shown)

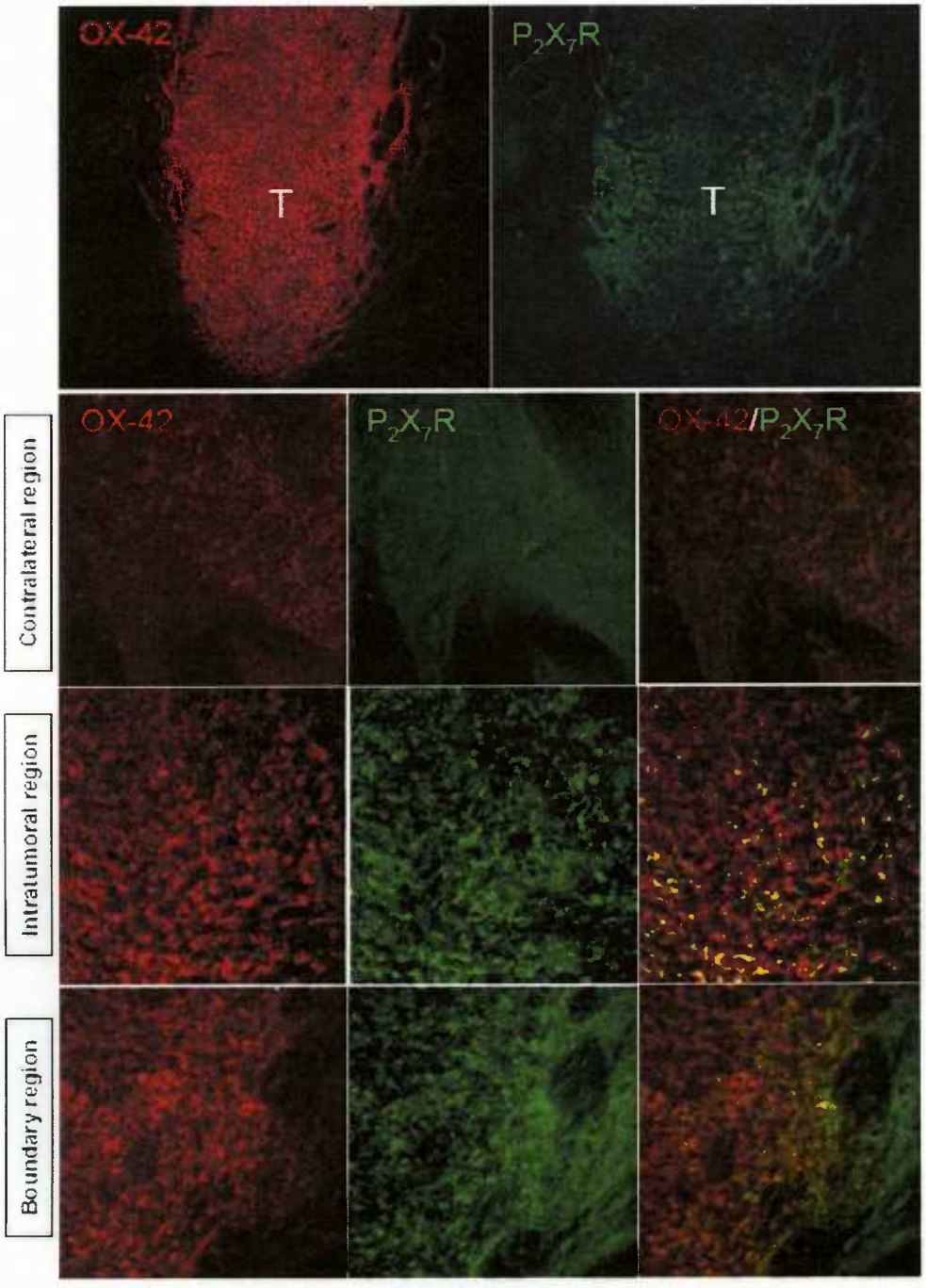


Figure 3-8. Microglia and P2X$_7$R stain throughout tumor
A high degree of co-localization of microglia and P2X$_7$R is found within intratumoral regions. For boundary region, both OX-42 and P2X$_7$R show ir in intratumoral, but not peritumoral areas. T is standing for tumor.

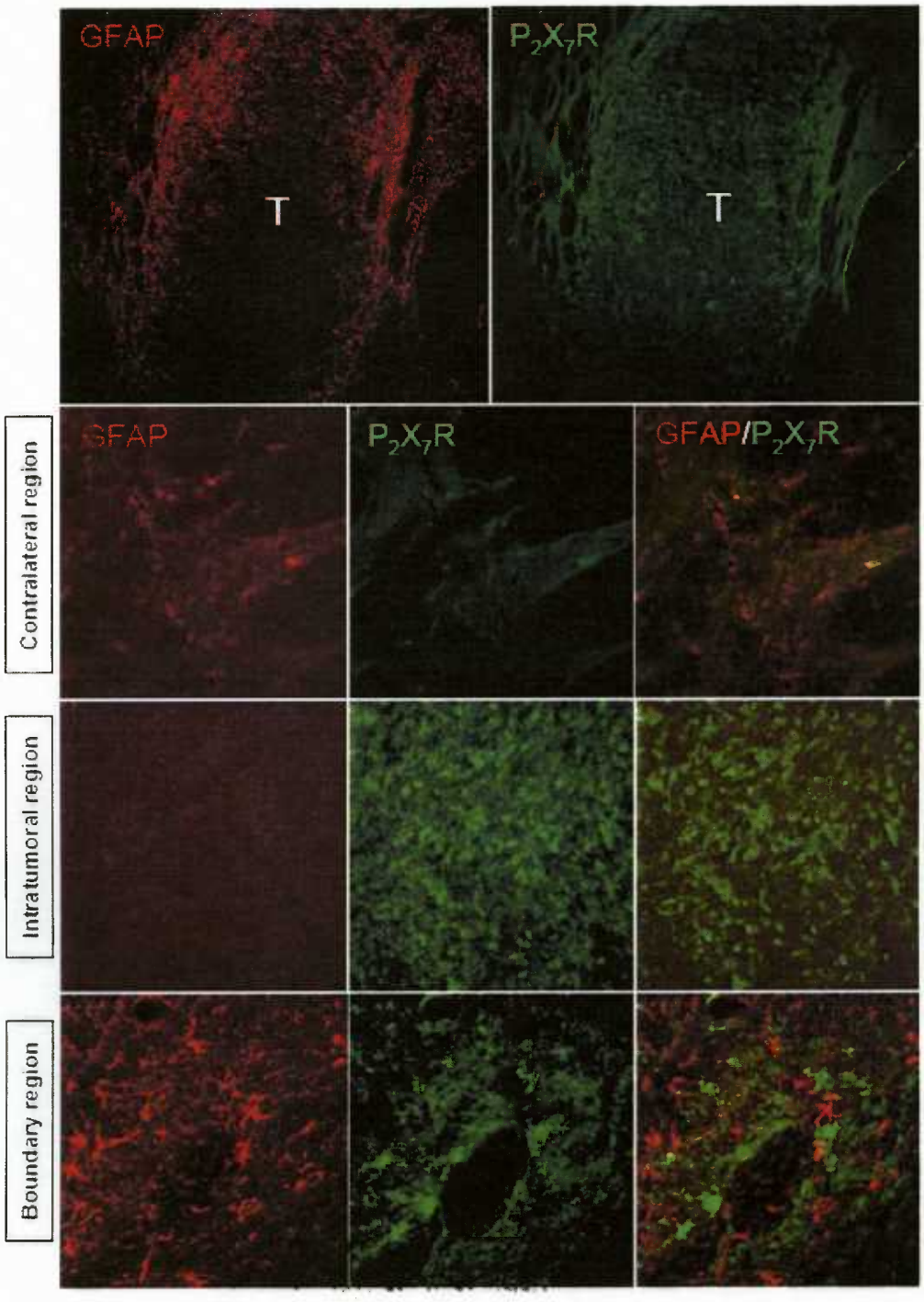


Figure 3-9. Astrocytes and $P2X_7R$ stain throughout tumor
GFAP staining is minimal in intratumoral regions which contain high levels of $P2X_7R$. Astrocytes are co-localized with $P2X_7R$ in peritumoral regions.

3.2.4. Administration of BBG reduced the tumor size

The $P2X_7R$ antagonist BBG was used in our study to inhibit $P2X_7R$ expressed in the tumor brain. Figure 3-10A shows serial coronal sections from tumor bearing animals injected only with saline (C6) or with BBG (C6 + BBG). Administration of BBG markedly reduced the size of brain tumors formed by transplanted C6 cells compared with saline injection. Quantification of the preliminary results (from n = 3 animals per group) is presented in Figure 3-10B and shows the tumor volume was reduced by about 65 % with BBG administration.

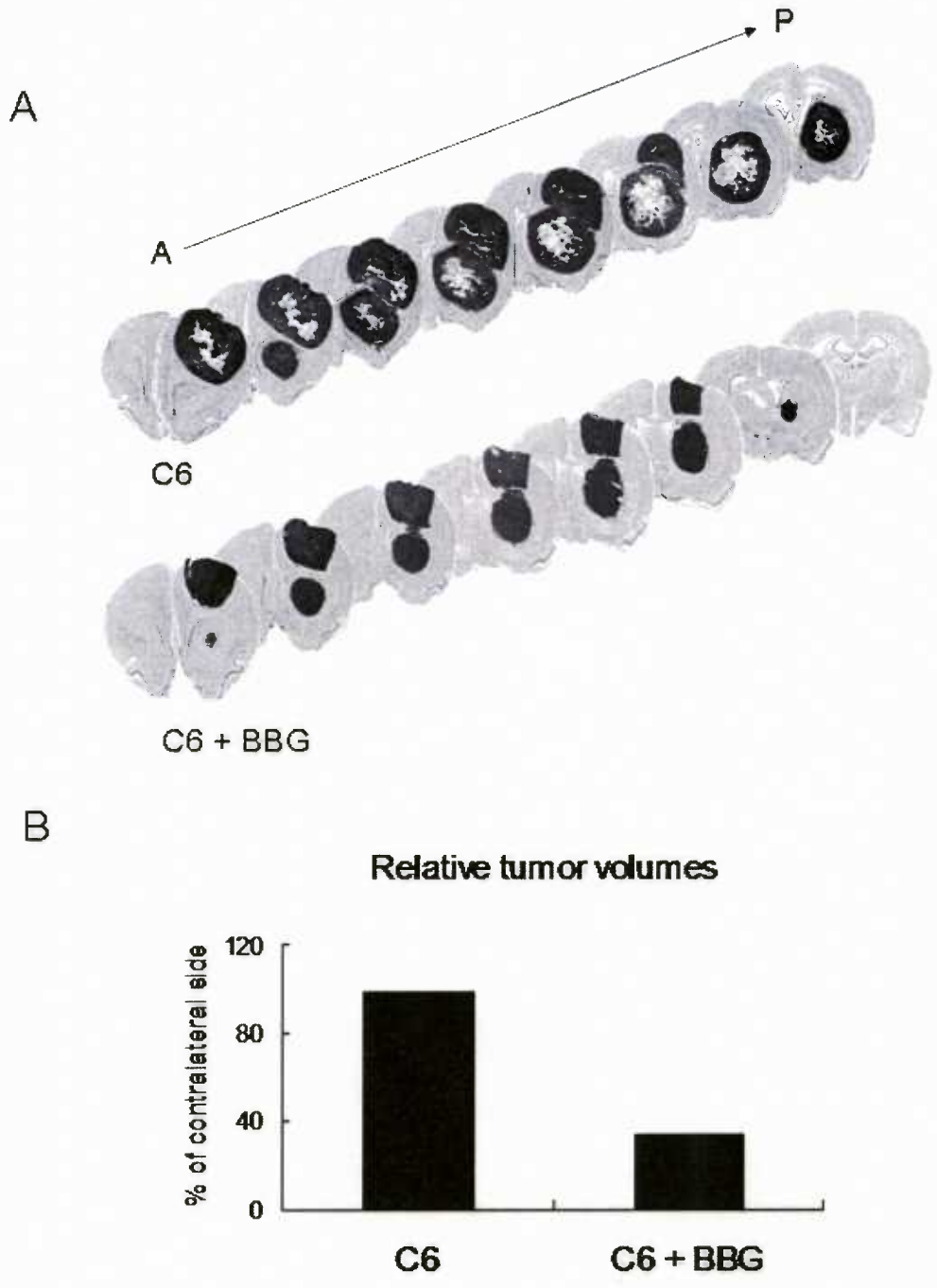


Figure 3-10. Administration of BBG reduced the tumor size

(A) Serial coronal sections from tumor bearing animals injected only with saline (C6) or with BBG (C6 + BBG). In both animals, the left hemisphere did not receive any surgery and served as the histological control. Nissl staining indicates tumor progression. A: anterior; P: posterior. (B) Quantification of tumor volumes. The relative tumor volume is expressed as the percentage of the absolute tumor volume to the volume of the intact hemisphere. n = 3 animals per group.

4. Discussion

4.1. In vitro studies

Our *in vitro* studies provide the first report for expression, at both mRNA and protein levels, of the purinergic subtype receptor, $P2X_7R$, in C6 glioma. Activation of this receptor using the specific ligand BzATP induced a rapid transient increase in $[Ca^{2+}]i$ which was sensitive to pretreatment of cells with the $P2X_7R$ antagonist, OxATP. Functional responses mediated by $P2X_7R$ in C6 cells were also demonstrated in this work. These included increased expression of pro-inflammatory factors, enhanced migration of cells and pore formation in the cell membrane; these responses are considered in more detail below.

In calcium imaging, BzATP induced a rapid increase in intracellular levels of Ca^{2+} followed by a slowly decreasing phase to basal level. This increase in $[Ca^{2+}]i$ following application of BzATP was partially, but not completely, antagonized by OxATP. Interestingly, we also observed a small increase in $[Ca^{2+}]i$ when BzATP was applied in a Ca^{2+}-free solution (data not shown). These findings indicate additional actions for BzATP in C6 glioma other than these mediated by $P2X_7R$. One possibility is that this agent also activated purinergic receptors of the metabotropic P2YR type. Some evidence is available indicating expression of $P2Y_2$ receptors ($P2Y_2R$) in C6 cells (Sabala et al., 2001) and our RT-PCR results also showed expression of this subtype P2YR in the present work (data not shown). Furthermore, BzATP has been shown as an activator of cloned human and rat $P2Y_2$ receptors (Erb et al., 1993; Wildman et al., 2003).

Previous studies have reported that $P2X_7R$ expression in cells other than C6 are up-regulated under pathological conditions and can mediate inflammatory responses (Franke et al., 2004; Lister et al., 2007; McLarnon et al., 2006; Yiangou et al., 2006). The present data suggest that activation of $P2X_7R$ in C6 glioma is associated with enhanced cellular expression of several pro-inflammatory factors including MCP-1, IL-8 and VEGF. Although the up-regulation of these factors in C6 cells was examined for a single duration of BzATP stimulation (8 hr), it is likely each would exhibit its own specific pattern of time changes with $P2X_7R$ activation. The factors could also act on differential targets. For example, expression of MCP-1 in malignant gliomas is closely associated with microglial and macrophage infiltration (Kielian et al., 2002; Platten et al., 2003). Although the functional responses of infiltrating immune cells are not well-understood, there is some evidence suggesting that these cells can contribute to the overall growth and invasion of brain tumors (Graeber et al., 2002; Wattere et al., 2005). Both VEGF and IL-8 are potent activators of angiogenic activity for vascular cells (Brat et al., 2005; Grobben et al., 2002) and these agents have reported actions to promote tumor neovascularity and progression (Brat et al., 2005; Grobben et al., 2002). Overall, our results are consistent with the idea that $P2X_7R$ in C6 cells could enhance tumor development by increasing tumor-associated inflammation and angiogenesis.

Activation of $P2X_7R$ was also linked to other functional responses including enhancement of cell migration in a scratch-wound assay and pore induction in a dye loading protocol. In the former case, increased mobility of C6 cells could aid migration of cells in a tumor environment. Recent work has reported extracellular

signal-regulated kinase (ERK) is activated downstream of $P2X_7R$ (Panenka et al., 2001) and is involved in migration of C6 glioma cells (Lind et al., 2006). Although pore formation is considered as a common trigger of cell death (Bulanova et al., 2005; Virginio et al., 1999a), we found no evidence for any cell damage with BzATP stimulation of C6 cells. Recent work using human neuroblastoma cell lines has reported that the two events are not necessarily linked (Raffaghello et al., 2006). $P2X_7R$ activation in neuroblastoma cells led to uptake of the YO-PRO dye without causing apoptosis or necrosis; indeed $P2X_7R$ acted as a growth-promoting factor in these cells. It is possible that in some cell types negative (death inducing) responses linked to $P2X_7$ activation have been modified to fit other functions; in C6, for example, it could link purinergic activation with pro-inflammatory responses. In addition, it should be noted that $P2X_2$ and $P2X_4$ receptors can also form large pores with longer term application of ATP (10-60 seconds) (Virginio et al., 1999b). However, the present results (Fig. 4) show that the $P2X_7R$ antagonist OxATP effectively blocked BzATP-induced dye uptake. This result suggests that $P2X_2$ or $P2X_4$ receptors do not participate in pore formation induced by BzATP.

Our work did not address the specific intracellular pathways linking activation of $P2X_7R$ with the specific cellular functional responses. It should be noted that although OxATP inhibited the increased $[Ca^{2+}]i$ induced by BzATP and also reduced migration of C6 cells it does not constitute definitive proof these processes are linked. Interestingly, OxATP treatment (300 μM for 8 hr) was ineffective in blocking the BzATP-induced increase in expression of the pro-inflammatory factors MCP-1, IL-8 and VEGF. A similar result was obtained with different application times of OxATP

(data not shown), suggesting that time of exposure of the $P2X_7R$ antagonist was not a factor involved. Our results could indicate that expression of the pro-inflammatory factors is mediated by Ca^{2+}-independent pathways coupled to activation of $P2X_7R$. However, as noted above, OxATP did not completely block $[Ca^{2+}]i$ induced by BzATP. Purinergic receptors other than $P2X_7R$ could also contribute to C6 responses in this study. As discussed above, $P2Y_2R$ can be expressed in C6 cells and could account for the residual calcium response induced by BzATP in the presence of the $P2X_7R$ antagonist, OxATP. In addition, BzATP has been reported as an agonist for several P2X receptor subtypes including $P2X_1R$ (Duan et al., 2003). Although our data report $P2X_7R$-mediated signaling in C6 cells, it is possible that other P2XR family members could be expressed in these cells.

In addition, it should be noted that in the migration study, C6 cells were cultured in media devoid of serum for 48 hours. This procedure was employed to minimize the effects of growth factors on cell mobility. However, it is possible that serum-free treatment could also alter the biological behavior of the cells. It has been reported that long-term serum starvation (up to 96 hrs) had effects on cell properties such as morphology and the adhesion pattern and expression of purinergic P2Y receptors in C6 glioma cells (Krzeminski et al., 2007).

Finally, it is important to recognize that no currently available animal tumor model would be expected to simulate human gliomas and species differences could affect expression and functions of the $P2X_7R$ in rat and human glioma cells. In addition, the properties of glioma cells derived from different tumors vary from each other so that data derived from a nitrosurea-induced tumor may not be valid for all gliomas.

Despite these limitations, the C6 glioma model has provided a wealth of information on *in vitro* and *in vivo* biochemical and biological properties of brain tumors (Barth, 1998).

Overall, our *in vitro* work has found C6 glioma cells express $P2X_7$ receptors which mediate Ca^{2+} mobilization and formation of large pores. Activation of the $P2X_7R$ is linked with cell expression of pro-inflammatory factors and cell migration which could contribute to tumor development.

4.2. In vivo studies

The preliminary *in vivo* studies showed a majority of $P2X_7R$ were associated with C6 glioma cells and microglia/macrophages (referred to only as microglia below). We also found pharmacological modulation of $P2X_7R$ with administration of the receptor antagonist Brilliant Blue G (BBG) markedly reduced tumor size.

BBG was used in the *in vivo* studies due to the following reasons: (1) It is a potent and selective $P2X_7R$ antagonist having IC_{50} values of about 10 nM at the rat $P2X_7R$ (Jiang et al., 2000). (2) We have demonstrated that it can cross the blood-brain barrier (BBB; data not shown), which makes it easy to be applied to animals (i.p. injection). However, since BBG is a blue dye it can interfere with signals, the dye is not suitable for *in vitro* assays such as Ca^{2+} imaging experiments.

In the *in vitro* studies, we have found that activation of the $P2X_7R$ is linked with cell expression of pro-inflammatory factors and cell migration which could contribute to tumor development. Since the $P2X_7R$ is not only expressed by C6 glioma cells *in*

vitro but also *in vivo*, it could have functional roles in regulating tumor growth and migration in the rat brain. One important link between *in vitro* and *in vivo* studies is that BBG inhibits C6 cell migration induced by BzATP (see **3.1.5.**). Therefore, BBG might reduce tumor size at least partially by inhibiting cell migration associated with $P2X_7R$ activation.

It was also clearly demonstrated that $P2X_7R$ were expressed by tumor-associated microglia in intratumoral regions. The presence of macrophages and microglia was reported in both human and rat gliomas (Morioka et al., 1992; Roggendorf et al., 1996; Shinonaga et al., 1988; Streit, 1994; Wierzba-Bobrowicz et al., 1994) and we also observed marked microglial infiltration within tumors. Astrocytes appeared primarily to be localized to boundary regions (data not shown). Although microglia are thought to play a significant role in neuronal injury in CNS inflammatory diseases, their function in brain tumors is poorly understood. A number of substances produced by microglia may contribute to glioma proliferation, migration and angiogenesis and the $P2X_7R$ is functionally involved in release of several of these factors such as IL-1β and TNF-α (see **1.2.3.2** and **1.3.3.**). Hence, overall tumor progression and invasion could be reduced by blockade of $P2X_7R$-mediated activities of microglia.

In addition, it is important to note that BBG is also a commonly used dye to stain protein. Although there is no report on toxicity of BBG to cells, there is the possibility that the compound may have some non-specific effects since it can bind to protein.

According to our present data, we suggest that BBG inhibits tumor growth by acting on $P2X_7R$ in both C6 glioma cells and microglia. However, further investigation is required to confirm the effects of BBG administration on tumor

development and to study the specific roles of $P2X_7R$ in brain tumors.

5. Conclusions of thesis research and future directions

Our work has found C6 glioma cells express $P2X_7$ receptors which mediate Ca^{2+} mobilization and formation of large pores. Activation of the $P2X_7R$ is linked with cell expression of pro-inflammatory factors and cell migration which could contribute to tumor development. In addition, preliminary *in vivo* studies showed that C6 glioma cells and tumor-associated macrophages/microglia are major contributors to $P2X_7R$ expression in tumor-bearing rat brains. Importantly, BBG administration inhibited tumor growth.

The results summarized above demonstrate that the $P2X_7$ receptor is an important modulatory factor in C6 glioma. Furthermore, pharmacological modulation of $P2X_7R$ *in vivo* alters tumor development. Understanding the roles of $P2X_7R$ in glioma brain tumors may lead to a rational testing of novel therapeutic strategies to inhibit tumor growth.

Further research needs to be carried out to confirm the effects of BBG administration on tumor development. The role of $P2X_7R$ in tumor-bearing brains could be further investigated by examining tumor-associated pathological processes such as angiogenesis and inflammation. These studies will give us a better understanding of the overall functions of $P2X_7R$ in glioma brain tumors.

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