P2X\textsubscript{7} Mediates Superoxide Production in Primary Microglia and Is Up-regulated in a Transgenic Mouse Model of Alzheimer’s Disease*

Lav K. Parvathenani\textsuperscript{1}, Svetlana Tetyshnikova, Corinne R. Greco, Susan B. Roberts, Barbara Robertson, and Rand Posmantur

From the Neuroscience Drug Discovery, Pharmaceutical Research Institute, Bristol-Myers Squibb Co., Wallingford, Connecticut 06492

Primary rat microglia stimulated with either ATP or 2'- and 3'-O-(4-benzoylbenzoyl)-ATP (BzATP) release copious amounts of superoxide (O\textsubscript{2}\textsuperscript{-}). ATP and BzATP stimulate O\textsubscript{2}\textsuperscript{-} production through purinergic receptors, primarily the P2X\textsubscript{7} receptor. O\textsubscript{2}\textsuperscript{-} is produced through the activation of the NADPH oxidase. Although both p42/44 MAPK and p38 MAPK were activated rapidly in cells stimulated with BzATP, only pharmacological inhibition of p38 MAPK attenuated O\textsubscript{2}\textsuperscript{-} production. Furthermore, an inhibitor of phosphatidylinositol 3-kinase attenuated O\textsubscript{2}\textsuperscript{-} production to a greater extent than an inhibitor of p38 MAPK. Both ATP and BzATP stimulated microglia-induced cortical cell death indicating this pathway may contribute to neurodegeneration. Consistent with this hypothesis, P2X\textsubscript{7} receptor was specifically up-regulated around \beta-amyloid plaques in a mouse model of Alzheimer’s disease (Tg2576).

Activated microglia have been observed in patients suffering from both acute (stroke) and chronic (Alzheimer’s disease) neurological disorders (1, 2). Microglia are believed to contribute to the progression of Alzheimer’s disease (AD)\textsuperscript{3} because these cells can release pro-inflammatory substances known to induce neurotoxicity (3). Reactive oxygen intermediates (ROIs), one of several pro-inflammatory substances released by microglia (4), are likely to play a very important role in AD because hallmark modifications of ROI damage such as lipid peroxidation and nitrotyrosine conjugates are characteristic of post-mortem AD brains (3). Hence, pro-inflammatory stimuli that promote microglial ROI production might contribute to the pathogenesis of AD.

ATP is an important messenger in the brain and can be released from cells by both lytic and non-lytic mechanisms (5). ATP evokes a variety of biological responses in microglia (6–9). The effects of ATP are mediated through interactions with the P2 purinoceptors, broadly classified into P2Y metabotropic and P2X ionotropic receptors (10). The P2Y receptors are G protein-coupled and P2X receptors are ligand-gated ion channels (10). Whereas the P2Y receptors are responsible for Ca\textsuperscript{2+} release predominantly from intracellular stores, P2X receptors are responsible for Ca\textsuperscript{2+} influx from extracellular sources.

Microglia possess both P2Y and P2X receptors (11–13). The P2X\textsubscript{7} receptor is highly expressed by cells of the macrophage lineage, such as dendritic cells, alveolar macrophages, and microglia. Activation of the P2X\textsubscript{7} receptor is unique in triggering the formation of large nonselective membrane pores, permeable to molecules up to 900 Da which ultimately results in death of the cell (9, 14). ATP and ATP analogs have been used to characterize the role of P2 receptors in microglial activation. Micromolar concentrations of ATP are required to activate the P2Y receptors, whereas millimolar (1–5 mM) concentrations of ATP are required to activate the P2X receptors. The ATP analog BzATP is a selective agonist at the P2X receptor and does not bind P2Y receptors (15, 16). Oxidized ATP (oATP) is a specific antagonist of P2X\textsubscript{7} that binds irreversibly to the receptor and prevents its activation by ATP (17). In this study, these pharmacological tools were used to determine the purinergic receptors involved in O2\textsuperscript{-} production in microglia.

The P2X\textsubscript{7} receptor plays a role in the generation of superoxide in microglia. Our studies elucidate a putative signal transduction pathway that mediates this response. These studies also demonstrate that BzATP- and ATP-activated microglia can mediate neurotoxicity. Finally, a distinct alteration was detected in the staining pattern for P2X\textsubscript{7} receptor in a transgenic mouse model of AD, suggesting that P2X\textsubscript{7} receptor activation could play a contributing role in AD.

MATERIALS AND METHODS

Reagents—Reagents not specified otherwise were obtained from Sigma. PD98059, SB203580, LY294002, and DFI were obtained from Biomol (Plymouth Meeting, PA). p38 MAPK and p42/44 MAPK phospho-antibody kits were obtained from New England Biolabs (Beverly, MA). P2X and p67\textsuperscript{phox} antibodies were obtained from Pharmingen. Anti-CD45 was purchased from Serotec (Oxford, UK). Amplex red kit and Fluo-4 were from Molecular Probes (Eugene, OR). Hematoxylin kit obtained from Shandon, Inc. (Pittsburgh, PA). The Tg2576 transgenic mice overexpressing mutant APP (K670N,M671L) and control mice were purchased from the Mayo Clinic (Jacksonville, FL).

Isolation of Microglia—Rat microglia were prepared from 2-day-old Sprague-Dawley rat pups. The rat cortices were separated from meninges and minces, triturated, and centrifuged (200 \times g for 10 min) to remove dead cells. The pellet was resuspended in media and triturated, and two brains were transferred to a 175-mm\textsuperscript{3} flask containing medium and incubated at 37 °C, 95% relative humidity in a 5% CO\textsubscript{2} atmosphere. The medium was changed after 3–4 days and twice a week thereafter. Microglia were isolated on day 10 by shaking the flasks on an orbital shaker (VWR Scientific) at 125 rpm for 15 min. The supernatant was passed through a sterile nylon mesh (20 \mu m) (VWR Scientific), and cells

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‡ To whom correspondence should be addressed: Curagen Corp., 322 East Main St., Branford, CT 06405. Tel.: 203-871-4432; E-mail: bparvathenani@curagen.com

1 The abbreviations used are: AD, Alzheimer’s disease; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; BzATP, 2'- and 3'-O-(4-benzoylbenzoyl)-ATP; DPI, diphenyleneiodonium chloride; ERK, extracellular signal-regulated protein kinase; H\textsubscript{2}O\textsubscript{2}, hydrogen peroxide; IFN\textgamma, interferon-\gamma; LPS, lipopolysaccharide; MAPF, mitogen-activated protein kinase; O\textsubscript{2}\textsuperscript{-}, superoxide; oATP, oxidized ATP; PI3-K, phosphatidylinositol 3-kinase; PMA, phorbol 12-myristate 13-acetate; PPADS, pyridoxal-5-phosphate-6-azophenyl-2'-4'-disulfonic acid; ROI, reactive oxygen intermediates; TNF\alpha, tumor necrosis factor-\alpha; PIPES, 1,4-piperazinediethanesulfonic acid; GBAP, glial fibrillary acidic protein; APP, amyloid precursor protein.
were collected by centrifugation (200 × g for 10 min) and used the same day. The purity of the cultures was 98–100% as determined by immunostaining with ED-4 antibody.

Isolation of Cortical Neurons—Primary cortical cell cultures were prepared from embryos of timed pregnant Sprague-Dawley rats at E14 (18). Briefly, the cortex triturated in DNaSe/Pastease dissociation buffer was centrifuged and resuspended in PC-1 SF medium (BioWhittaker). The cells (2 × 10^6/ml) were plated onto poly-L-ornithine-coated 24-well plates, and 4 days later the media were replaced with Neurobasal Medium containing B-27 supplement (Invitrogen), 1% penicillin/streptomycin, and 10 mM t-glutamine. Neuronal cells constituted 90–95% of the total cells and were used on day 10 for experiments.

Isolation of Neutrophils—Neutrophils were isolated from peripheral blood by lysis of red blood cells donors as recommended previously (19). Measurement of Superoxide Production—Superoxide (O_2^-) was measured indirectly through the detection of hydrogen peroxide (H_2O_2) by the method of Mohanty et al. (20). O_2^- production was measured in initial experiments by O_2^-/dependent superoxide dismutase-sensitive reduction of ferricytochrome c (21). However, microglia released very little O_2^- and this procedure required a large number of cells. In subsequent experiments the more sensitive method of H_2O_2 detection using conversion of 10-aceetyl-3,7-diarylxylophenoxazine (Amplex Red) to highly fluorescent resorufin in the presence of horseradish peroxidase was followed (20). Briefly, 5 × 10^5/ml microglia in Hank’s balanced salt solution (HBSS) were preincubated with the inhibitors for either 2 (Table I) or 1 h (SB203580, PPADS). The H_2O_2 released was calculated as picomoles of H_2O_2, and the change in fluorescence was measured at 544 nm after excitation at 590 nm in a time-resolved mode (1-Hz frequency). Relative relaxation of ferricytochrome c was followed (20). Briefly, 5 × 10^5/ml microglia growing in LADMAC-conditioned media (ATCC, Manassas, VA) were loaded with Fluo-4, AM (5 μM) in HBSS containing 10 mM HEPES (pH 7.4) for 1 h before the experiment at room temperature and washed with HBSS. The mice were sacrificed (23). Briefly, microglia (1 × 10^5) in HBSS containing 10 mM HEPES (pH 7.4) for 1 h, 500 μM H_2O_2, and the resulting supernatant was designated the cytosolic fraction. In subsequent experiments the more sensitive method of H_2O_2 detection using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) to highly fluorescent resorufin in the presence of horseradish peroxidase was followed (20). Briefly, 5 × 10^5/ml microglia in Hank’s balanced salt solution (HBSS) were preincubated with the inhibitors for 2 h (SB203580, PPADS). The H_2O_2 released was calculated as picomoles of H_2O_2, and the change in fluorescence was measured at 590 nm after excitation at 544 nm every 2.5 min using a fluorometric plate reader (Fluostar, BMG Labtechnologies, Durham, NC). The H_2O_2 was estimated using the Bio-Rad DC Protein Assay, and 25 μg of protein was fractionated on a 10% SDS-PAGE gel, transferred to polyvinyllidine difluoride membrane, and blocked in 5% milk/Tri-buffered saline containing 0.1% Tween 20 for 2 h.

Immunoblotting—Immunoblotting was performed as described previously (18). Cells were homogenized in 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride, and 1:100 dilution of probe was generated using 10% SDS-PAGE gel, transferred to polyvinyllidine difluoride membrane, and blocked in 5% milk/Tri-buffered saline containing 0.1% Tween 20 for 2 h. The membranes were washed and incubated overnight with antibodies specific for phospho-p42/44 MAPK (Thr-202/Tyr-204), phospho-p38 MAPK (Thr-180/Tyr-182) diluted 1:1000 in TBST containing 5% bovine serum albumin. Membranes were incubated with an horseradish peroxidase-conjugated secondary antibody (1:2000) for 2 h. The membranes were washed extensively, and bands were detected using LumiGLO. The membranes were stripped using RESTORE Western blot stripping buffer (Pierce), washed several times, and blocked for 1 h. Membranes were incubated with antibodies specific for either unphosphorylated p42/44 MAPK or p38 MAPK diluted 1:1000 in blocking buffer. The day next membranes were incubated with the secondary antibody and visualized using LumiGLO.

The P2X, (1,500), p67phox, (1,500), and actin (1,750) antibodies were used according to the manufacturer’s recommendation. In some experiments a P2X, control peptide corresponding to amino acid 576–585 of rat P2X receptor was used to generate the antibodies to determine specificity of the bands. The P2X, antibody was preincubated with the control peptide at a 1:1 dilution (v/v) for 1 h at room temperature prior to the addition to the membrane.

Neurotoxicity Assay—Primary rat microglia (1 × 10^5) in Neurobasal Medium containing B-27, 1% penicillin/streptomycin, and 10 mM t-glutamine were seeded into a 48-well plate containing 1 × 10^5 primary cortical neurons. The cells were allowed to settle for 2 h prior to the addition of stimuli. After a 72-h incubation, the supernatant was assayed for lactate dehydrogenase (LDH). Microglia and cortical cells were also independently cultured for 72 h in the presence of stimuli, and LDH released from microglia alone + stimuli were subtracted out from the values obtained from the combination of microglia and cortical neurons. The LDH was measured with a commercial kit obtained from Promega (WI). In one experiment a WST-1 cell survival assay was performed on the cells remaining in the well with a commercial kit obtained from Roche Diagnostics. WST-1 is a modified 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. The WST-1 assay enables colorimetric measurement of cell viability based on the cleavage of tetrazolium salts by mitochondrial dehydrogenase in viable cells.

TNFα Enzyme-linked Immunosorbent Assay—Supernatants were assayed for TNFα using OPEIA Rat TNFα kit (Pharmping).

Nitrite Assay—Nitrite assay was performed in a 96-well plate using nitrated Cells per well (2-year Tg2576 mice and aged-matched controls) were removed and fixed in 4% paraformaldehyde. The brains were dehydrated in graded series of alcohols, washed in deionized water, and incubated in 100 μl of methanol containing 3% H_2O_2 for 30 min to quench endogenous peroxidase activity. The slides were rinsed in deionized water for 5 min followed by blocking in 5% normal goat serum in phosphate-buffered saline containing 0.01% Triton-X-100 for 1 h. Sections were incubated with primary antibody (Pan-αβ 1:1000 (QCB), 4G5 1:1000 (Signet), P2X, 1:100 (Pharmping), CD4 1:200 (Serotec), or others) incubated for 1 h at room temperature. After rinsing the secondary antibody (Vector Laboratories) was added, the manufacturer’s protocol was followed. The slides were washed in water and counter-stained with Shandon Lippshaw hematoxylin stain for 2 min at room temperature. Sections were washed in water for 1 min, incubated for 10 s in 50% ethanol/
H₂O₂ production in microglia was determined in 2 × 10⁵ microglia (D) or 1 × 10⁶ neutrophils (E) stimulated with BzATP (100–250 μM) or PMA (20 ng/ml) for 30 min. The data are the mean ± S.D. of triplicate samples repeated at least twice.

The maximal stimulus was −250 μM. The magnitude of the response was higher in cells treated with BzATP compared with ATP. The total amount of H₂O₂ produced (picomoles) with stimulation by ATP and BzATP was lower than that generated by 10 ng/ml phorbol 12-myristate 13-acetate (PMA). However, at early time points (5 min) 250 μM BzATP generated 9–10-fold more H₂O₂ than PMA (Fig. 1C). These results demonstrate there is a distinct difference in both the magnitude and duration of H₂O₂ production depending on the stimulus.

The conversion of Amplex Red to highly fluorescent resorufin in the presence of H₂O₂ is an indirect measure of O₂⁻ generation. Hence the production of O₂⁻ was confirmed by a more direct but less sensitive method. The inhibition of reduction of ferricytochrome c by O₂⁻-dependent superoxide dismutase was used to detect the generation of O₂⁻. As shown in Fig. 1D, microglia treated with BzATP showed a significant increase in superoxide dismutase-inhibitable reduction of ferricytochrome c compared with untreated microglia. O₂⁻ production by neutrophils treated with BzATP was examined as a control. Similar increases in O₂⁻ were observed when neutrophils were stimulated with BzATP suggesting that BzATP activates a similar cascade in both cell types.

To determine whether BzATP generated any intracellular O₂⁻, the reduction of NBT in neutrophils (Fig. 1E) suggested that BzATP activates a similar cascade in neutrophils (Fig. 1E). To determine whether BzATP generated any intracellular O₂⁻, the reduction of NBT in neutrophils treated with BzATP was examined. In neutrophils treated with BzATP or PMA but not control cells, formations of purple granules of formazan were visible microscopically indicating that NBT was being reduced to formazan (data not shown). These results confirm that cells stimulated with BzATP generated O₂⁻. Because microglia produce little O₂⁻, the conversion of Amplex Red to highly fluorescent resorufin, a more sensitive but indirect indicator of O₂⁻ production (20), was used as the choice reagent in the remaining experiments.

Effect of ATP and BzATP on Calcium—Treatment of microglia with ATP or BzATP resulted in a very rapid increase in the
FIG. 2. Intracellular free Ca$^{2+}$ changes in cells treated with ATP and BzATP in the presence and absence of extracellular Ca$^{2+}$. Primary rat microglia (1 × 10$^4$) were plated onto a 384-well plate and stimulated 10 s after beginning the experiment with various concentrations of ATP or BzATP in HBSS (A), various concentrations of BzATP in HBSS without Ca$^{2+}$ or Mg$^{2+}$ containing 0.5 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (C). The response was measured using a fluorescence plate reader. The black lines represent responses in the presence and the gray lines represent responses in the absence of Ca$^{2+}/$Mg$^{2+}$ ions. The data are the means of quadruplicate samples repeated at least twice.

level of intracellular calcium (Fig. 2). ATP stimulated a transient increase of intracellular calcium (Fig. 2A). BzATP caused a sustained increase in the level of intracellular free calcium ([Ca$^{2+}$]$^i$) that was maintained for more than 6 min (Fig. 2B). The concentration of ATP or BzATP required for maximal [Ca$^{2+}$]$^i$ change was lower than required for maximal H$_2$O$_2$ production. Maximal [Ca$^{2+}$]$^i$ changes were stimulated by 30-100 μM of both ATP and BzATP, whereas maximal ROI production required 1 mM ATP and 250 μM BzATP (Figs. 1 and 2).

To determine whether ATP and BzATP were mobilizing intracellular or extracellular sources of Ca$^{2+}$ or both, additional experiments were carried out in Ca$^{2+}$-free media containing 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (Ca$^{2+}$ chelator). In the absence of extracellular Ca$^{2+}$, the BzATP response was completely blocked indicating that BzATP was mobilizing only extracellular Ca$^{2+}$ (Fig. 2C). However, with ATP, the initial transient peak was reduced by about 75%, suggesting that ATP mobilizes both intracellular (via inositol 1,4,5-trisphosphate-induced Ca$^{2+}$ release) and extracellular sources of Ca$^{2+}$ (possibly via capacitative Ca$^{2+}$ influx) (24, 25).

Effect of Extracellular Calcium on ROI Production—Because ATP appeared to stimulate Ca$^{2+}$ release from intracellular stores and Ca$^{2+}$ influx from extracellular sources, whereas BzATP appeared to stimulate only Ca$^{2+}$ influx from extracellular sources, the effect of removal of extracellular Ca$^{2+}$ on H$_2$O$_2$ production was examined. Both ATP- and BzATP-stimulated H$_2$O$_2$ production was blocked to below control levels in the absence of extracellular Ca$^{2+}$ (Fig. 3). These results suggest that despite the differences in Ca$^{2+}$ mobilization, both ATP and BzATP required only extracellular Ca$^{2+}$ to generate H$_2$O$_2$.

Receptors Involved in the Generation of ROI—The ability of BzATP, an agonist of P2X receptors, to stimulate H$_2$O$_2$ production and the requirement of extracellular Ca$^{2+}$ for this response suggest P2X receptors mediate the production of H$_2$O$_2$ in microglia. To determine whether the production of H$_2$O$_2$ was mediated through the P2X$_7$ receptor, two selective inhibitors of P2X$_7$, PPADS and oATP, were tested. Both PPADS and oATP blocked H$_2$O$_2$ production by BzATP treatment (Fig. 4A) suggesting that BzATP activates H$_2$O$_2$ production primarily through the P2X$_7$ receptor. Further support for the role of P2X$_7$ in H$_2$O$_2$ production was obtained by treating cells with Brilliant Blue G, a potent and highly selective inhibitor of P2X$_7$, at nanomolar concentrations (26). Brilliant Blue G (500 nM) inhibited BzATP (250 μM)-induced H$_2$O$_2$ production by more than 80% (Fig. 4A).

To determine whether αTP and PPADS affected Ca$^{2+}$ responses similarly, Ca$^{2+}$ changes were measured in cells pretreated with αTP and PPADS in the presence or absence of BzATP. αTP (100 μM) inhibited BzATP-induced Ca$^{2+}$ flux to near control levels (Fig. 4B). Similar results were obtained with PPADS (Fig. 4B). These results suggest that P2X$_7$ is the primary receptor stimulated by BzATP to generate H$_2$O$_2$.

Source of ROI—Several sources can contribute to the production of ROI. These include the classical NADPH oxidase, the mitochondrial respiratory chain, and microsomal enzymes. Pharmacological inhibitors of the NADPH oxidase were used to determine whether P2X$_7$ receptor activates NADPH oxidase. Three selective inhibitors with different mechanisms of action, diphenyleneiodonium chloride (DPI), apocynin, and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) were used (27, 28). As shown in Fig. 5A, all three inhibitors completely inhibited BzATP-induced H$_2$O$_2$ release from microglia. To confirm the activation of NADPH oxidase by BzATP in microglia, a functional change in NADPH oxidase was examined. A critical step in the activation of the NADPH oxidase is the translocation of p67$^{phox}$ from the cytosol to the membrane. In BzATP-stimulated microglia, p67$^{phox}$, which is primarily cytosolic, rapidly translocated to the particulate/membrane fraction (Fig. 5B). These results suggest that BzATP stimulates the release of ROI in microglia via the activation of the NADPH oxidase.

Signal Transduction Cascade Involved in the Generation of H$_2$O$_2$—The production of O$_2^-$/H$_2$O$_2$ is stimulated through several different signal transduction pathways. Two kinases implicated in the activation of the NADPH oxidase are p42/44 ERK and p38 MAPK (29–31). Both p42/44 ERK and p38 MAPK were rapidly activated in cells stimulated with 250 μM BzATP (Fig. 6). However, pretreatment with SB203580 (a selective p38 MAPK inhibitor) but not PD98059 (a selective p42/44 MAPK inhibitor) attenuated H$_2$O$_2$ production in cells stimulated with...
MAPK attenuates H2O2 production. Furthermore, PI3-K may inhibit H2O2 production by 74.7% for BzATP-induced H2O2 production and calcium changes. 

Primary rat microglia (1 × 10⁶) were plated and pretreated with oATP or PPADS for 2 h or Brilliant Blue-G (BB) for 1 h. H2O2 production (A) or Ca²⁺ changes (B) were measured in cells treated with BzATP. The data are the means ± S.E. of five (PPADS, oATP) or three (Brilliant Blue-G (BB)) experiments each done in quadruplicate. The data are presented as a percent of H2O2 produced by microglia stimulated with 250 μM BzATP for 30 min. * indicates p < 0.05 by Student’s t test.

BzATP (Fig. 6). Nonetheless, PD98059 did attenuate BzATP-induced TNFα release suggesting that p42/44 MAPK was involved in cytokine signaling but not in the generation of H2O2 (data not shown). LY294002, a selective phosphatidylinositol 3-kinase inhibitor (PI3-K) also significantly inhibited H2O2 release (data not shown). LY294002, a selective phosphatidylinositol 3-kinase inhibitor (PI3-K) also significantly inhibited H2O2 release (data not shown). The inhibitors used were not toxic to the cells at concentrations used. These results suggest that whereas both p38 MAPK and p42/44 ERK are rapidly activated in microglia stimulated with BzATP, only the inhibition of p38 MAPK attenuates H2O2 production. Furthermore, P38-K may play a more important role in the release of H2O2 than p38 MAPK because the PI3-K inhibitor blocks H2O2 release to a greater extent than the p38 MAPK inhibitor.

Microglia Stimulated with ATP or BzATP Are Neurotoxic—To determine whether activation of microglia with ATP and BzATP is neurotoxic, a co-culture system using highly purified primary rat cortical neurons and primary rat microglia was employed. Stimulation of microglia with LPS resulted in massive production of TNFα and nitric oxide but no H2O2 release (Figs. 1 and 7, B and C). LPS did not stimulate LDH release from microglia/cortical neurons co-cultures (Fig. 7A). Conversely, ATP or BzATP stimulated very little TNFα and nitric oxide production but induced neurotoxicity at 72 h (Fig. 7). There was no significant neurotoxicity up to 48 h post-stimulation with either ATP or BzATP. The amount of neurotoxicity was much greater with BzATP compared with ATP, which is consistent with the observation that BzATP is a more potent stimulus compared with ATP in the generation of H2O2. The values shown in Fig. 7A represent LDH released from both microglia and cortical cells minus LDH released from microglia alone. A WST-1 cell survival assay was used to confirm that the LDH release is a measure of neuronal toxicity. Neither ATP nor BzATP had a significant neurotoxic effect on cortical neurons (Fig. 7A). Results comparing LPS-treated co-cultures to ATP/BzATP-treated co-cultures show that factors other than TNFα or nitric oxide are contributing to toxicity in our system.

Up-regulation of P2X7 Receptor in a Transgenic Mouse Model of Alzheimer’s Disease—By having demonstrated that ATP- and BzATP-stimulated microglia release H2O2 and kill cortical neurons in vitro, we examined the brains of a transgenic mouse model of AD (Tg2576 carrying a APPK670N,M671L double mutation) (32) to determine whether the mechanism is important in vivo. In 24-month-old transgenic mouse brains but not in aged-matched control brains, plaques were evident in the hippocampus and surrounding outer cortical region when stained with two different antibodies for Aβ (Fig. 8, A, B, and E; data not shown). P2X7, immunostaining gave a ring-like pattern around plaques only in transgenic mice suggesting that cells staining for P2X7 were surrounding the plaques (Fig. 8, C, D, and F). This staining was not evident in the absence of the primary antibody indicating that the staining was not due to nonspecific binding of the secondary antibody to the plaques. There was some basal staining with the P2X7 antibody in both control and transgenic animals suggesting low levels of P2X7 are expressed in the brain.

To determine whether the increased staining of P2X7 in the transgenic mice was due to increased expression of P2X7 receptor, lysates from the hippocampi (region with higher concentration of plaques) of three 19-month Tg2576 mice and

![Fig. 4. Effect of P2X selective inhibitor oATP and PPADS on BzATP-induced H2O2 production and calcium changes.](image-url)

![Fig. 5. NADPH oxidase is activated by BzATP. A, primary rat microglia (1 × 10⁶) were plated and pretreated with AEBSF, apocynin, or DPI for 30 min, and H2O2 production was measured in cells stimulated with 250 μM BzATP. The data are the means ± S.D. of quadruplicate samples repeated three times. * indicates p < 0.05 by Student’s t test. B, microglia were left untreated or activated with 500 μM BzATP for 5–10 min. The cells were then collected and fractionated as described under “Materials and Methods.” p67phox was detected using a polyclonal antibody.)](image-url)
Activation of P2X7 Stimulates Superoxide Production

The signal transduction cascade activated by BzATP in the generation of H2O2 in primary rat microglia (1 × 10^5) were plated and pretreated with SB203580, PD98059, or LY294002 for 1 h, and then H2O2 production was measured in cells stimulated with 250 μM BzATP. The data represent the means ± S.E. of three experiments each done in triplicate. The data are presented as a percent of H2O2 produced by microglia stimulated with 250 μM BzATP for 30 min. * indicates p < 0.05 by Student’s t test. Lysates of cells stimulated with 250 μM BzATP or 100 ng/ml of LPS for various periods were probed with antibodies against phosphorylated p38 or unphosphorylated p38 MAPK antibodies (B) against phosphorylated p42/44 MAPK or unphosphorylated p42/44 MAPK (C).

DISCUSSION

The P2X7 receptor has been implicated in the activation of transcription factors, apoptosis, and in the release of pro-inflammatory substances like TNFα and interleukin-1β in microglia (8, 9, 14, 33). In this report we demonstrate that P2X7 is the primary receptor involved in H2O2 production in primary rat microglia stimulated with ATP or BzATP. The P2X-selective agonist BzATP was a more potent stimulus than ATP, a P2Y/P2X agonist. Functionally, the activation of microglia with ATP or BzATP induced cell death in primary cortical neurons. In vivo there was a striking association of P2X7 receptor-positive cells around plaques in a transgenic mouse model of Alzheimer’s disease.

No detailed study has profiled the expression of P2Y and P2X receptors in microglia, but microglial expression of both P2X and P2Y is supported by electrophysiological studies (13, 34). Our results demonstrating Ca2+ changes induced by ATP or BzATP also support the existence of functional P2X and P2Y receptors on primary rat microglia.

Several lines of evidence point to P2X7 receptors and P2X7, in particular, as the primary receptor involved in the generation of H2O2 in ATP- or BzATP-stimulated microglia. Stimulation of H2O2 production by P2X-selective agonist BzATP provides the first line of evidence for the involvement of P2X receptors. P2X receptors mobilize only extracellular Ca2+, consequently the experiments that demonstrate BzATP mobilizes only extracellular Ca2+ provide further evidence for the involvement of this receptor. The inability of BzATP to stimulate H2O2 production in the absence of extracellular Ca2+ provides a direct link between Ca2+ mobilization and H2O2 production. The inhibition of H2O2 production and Ca2+ influx by P2X-selective antagonist PPADS, P2X-selective antagonist oATP (17), and P2X7-selective inhibitor Brilliant Blue-G in BzATP-stimulated cells provide additional lines of evidence for the involvement of P2X7 receptors. The contribution of other purinergic receptors cannot be excluded, and can be evaluated only by measuring H2O2 production in microglia lacking P2X7 receptors.
The levels of ATP required to stimulate the P2X7 receptor in vitro suggests that the low concentrations of ATP found in the extracellular milieu of the brain would not be sufficient for microglia to induce neurotoxicity. However, recent reports (6) suggest that low concentrations of ATP can act as a chemoattractant for microglia directing them to a region of injury. Furthermore, ATP released from activated astrocytes has been shown recently (37) to activate microglia. Finally, a recent report (38) has documented the significance, the source, and needed high concentrations of ATP to activate P2X7 receptors in the brain, as well as the role of ATP in neurodegeneration.

Endogenous production of O2 is a biochemical process that requires tight regulation. NADPH oxidase, a known generator of O2, is regulated by an intricate signal transduction cascade within cell types such as neutrophils (39). Dysregulation of the oxidase leads to the damage of surrounding tissue and results in inflammatory conditions. Both p42/44 ERK and p38 MAPK play an important role in the regulation of various proteins of NADPH oxidase complex in neutrophils (29–31). However, little is known about the regulation of the NADPH oxidase in microglia. Although both p42/44 ERK and p38 MAPK were activated very rapidly in microglia stimulated with BzATP, only inhibition of p38 MAPK attenuated the release of H2O2. This suggests that there are subtle differences in the regulation of the NADPH oxidase complex in neutrophils compared with microglia. Other kinases like PI3-K promote NADPH oxidase activity through the PX domain of p47phox and p40phox (40). Inhibition of H2O2 levels by the PI3-K inhibitor, LY294002, suggests that PI3-K plays a similar yet more important role in the assembly of the oxidase complex in microglia compared with p38 MAPK.

Microglia activated by certain stimuli can induce cell death in cortical neurons. Although the neurotoxicity is attributed to various individual secretory products like TNFα, nitric oxide,
and ROIs, toxicity is likely due to a combination of factors (41–43). The inability of LPS-activated microglia to induce cell death in cortical neurons despite large increases in TNFα and inducible nitric-oxide synthase shows that these products do not solely induce cell death in our microglia/cortical neuron co-culture system. Published reports support this observation. Klegeris et al. (44, 45) demonstrated that LPS-stimulated microglia are not neurotoxic but in combination with IFNγ induces neurotoxicity. IFNγ can prime microglia to generate O₂⁻ in the absence and presence of TNFα suggesting that TNFα could be indirectly inducing cell death by generating O₂⁻ (46, 47). Moreover, both IFNγ/LPS or IFNγ/TNFα can up-regulate P2X₇ expression in THP-1 cells and monocytes (48, 49). Given that ROIs can directly induce neurotoxicity, it will be important to determine the exact mechanism by which ATP and BzATP are inducing cortical cell death. The link between LPS, IFNγ, and P2X7 expression has yet to be examined in detail in microglia.

Currently there are no reports on the expression profile of P2X7 in Alzheimer’s disease. Ours is the first paper demonstrating a remarkable difference in the staining pattern for P2X7 in brain slices of a transgenic (Tg2576) mouse model. This intense staining for P2X7 around plaques can be the result of up-regulation of the P2X7 receptor and/or aggregation of glia around plaques. The fact that P2X7 message and receptor is up-regulated in monocytes treated with LPS/IFNγ or LPS/TNFα (48, 50) raises the possibility that P2X7, indeed could be up-regulated in the mouse model of AD. The increased P2X7 immunoreactivity in immunoblots comparing Tg2576 hippocampi to age-matched control hippocampi lysates and the presence of P2X7-immunopositive cells around plaques supports this theory. However, the identity of the P2X7 immunopositive cells around plaques is still not clear, even though activated microglia and astrocytes are found in the same vicinity. Although a correlation has been established between increased P2X7 immunoreactivity and amyloid plaques, the question of cause and effect is beyond the scope of this paper. Because P2X7 knock out mice have been made, it will be interesting to see if microglia from these mice generate H₂O₂ in response to BzATP or if a P2X7 knock out mouse crossed with a Tg2576 would have any alteration in plaque deposition. It is possible that receptor antagonists of P2X7 could have therapeutic utility in treatment of AD by regulating pathologically activated microglia.

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Activation of P2X7 Stimulates Superoxide Production
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Lav K. Parvathenani, Svetlana Tertyshnikova, Corinne R. Greco, Susan B. Roberts, Barbara Robertson and Rand Posmantur

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