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Purine Release from Spinal Cord Microglia after Elevation of Calcium by Glutamate

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ABSTRACT

The propagation of Ca^{2+} waves in a network of microglial cells, after its initiation by glutamate, is mediated by purinergic transmission. In this study, we investigated the mechanisms by which glutamate releases ATP from cultured spinal cord microglia. The 4-fold increase in ATP release from microglia in response to glutamate (0.5 mM) was blocked by α-amino-hydroxy-5-methyl-isoxazole-4-proprionate (AMPA)/kainate receptor antagonist 6-cyano-7-nitroguinoxaline-2,3-dione and specific AMPA receptor antagonist 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride (GYKI 52466) but not by *N*-methyl-D-aspartic acid or metabotropic glutamate receptor antagonists. Glutamate acting on AMPA receptors evoked an ATP release that was blocked by antagonizing the rise in intracellular Ca^{2+} as a result of its release from internal stores as well as by antagonizing protein kinase C with chelerythrine. Glutamate-stimulated ATP release was significantly antagonized by the cystic fibrosis transmembrane conductance regulator (CFTR) blockers flufenamic acid and glibenclamide. A role for the CFTR was further confirmed using microglia from CFTR knockout mice, which released significantly less ATP than microglia from control wild-type mice in response to glutamate. Use of 6-methoxy-1-(3-sulfopropyl)quinolinium fluorescence assay revealed functional CFTR in microglia. These observations suggest that glutamate acted on microglial AMPA receptors to stimulate release of Ca^{2+} from intracellular stores as well as a Ca^{2+}-dependent isoform of protein kinase C, which then acts to trigger release of ATP with the CFTR acting as a regulator of the ATP release process, perhaps through another channel or transporter.

Purines released extracellularly exert several physiological and pathological effects that are mediated by microglia. Purinergic mechanisms have been shown to control the rapid extension and retraction of fine microglial processes that occur in the brain as well as the development of microglial processes directed toward regions of damaged tissue in the brain (Davalos et al., 2005). Both the baseline motility of the microglial processes and their response to injury of nearby tissue involves an action of ATP and possibly other purines on the metabotropic P2Y class of receptors on the microglia (Davalos et al., 2005), which are known to possess P2Y1, P2Y2, P2Y4, and P2Y12 receptors (James and Butt, 2002). It has also been shown recently that if the ionotropic P2X7 receptor, uniquely found in microglia and Schwann cells (Sim

et al., 2004), is knocked out in mice, they no longer experience either inflammatory or neuropathic pain (Chessell et al., 2005). Also P2X4 receptors are up-regulated in microglia during nerve-injury induced pain, acting in this case as a necessary molecular mediator (Tsuda et al., 2005). Furthermore, activation of P2X7 receptors on microglia are known to potentially lead to their release of pro-inflammatory cytokines (Hide, 2003; Suzuki et al., 2004) and activation of P2Y1 and P2Y2/4 can lead to the release of anti-inflammatory cytokines (Seo et al., 2004).

The question arises as to the identity of the principal sources of extracellular ATP in the basal state and after injury that could regulate the multifarious functions attributed to activation of P2 receptors on microglia. It is well established that astrocytes are one such source in that they use ATP as a principal transmitter for propagation of Ca^{2+} waves in the astrocyte network (Wang et al., 2000; Bennett et al., 2005). Microglia are known to release ATP (Ballerini et

ABBREVIATIONS: AMPA, α-amino-hydroxy-5-methyl-isoxazole-4-proprionate; NMDA, *N*-methyl-D-aspartic acid; DMEM, Dulbecco's modified Eagle's medium; LPS, lipopolysaccharide; FITC, fluorescein isothiocyanate; CFTR, cystic fibrosis transmembrane conductance regulator; ACPD, (1S,3R)-1-amino-cyclopentane-1,3-dicarboxylic acid; GDPβS, guanosine 5'-O-(2-thio)diphosphate; GFAP, glial fibrillary acidic protein; CNQX, 6-cyano-7-nitroguinoxaline-2,3-dione; SPQ, 6-methoxy-1-(3-sulfopropyl)quinolinium; GYKI 52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride; CTZ, cyclothiazide; PEPA, 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluorophenoxyacetamide; PTX, pertussis toxin; PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; NMDG, diisothiocyanstilbene disulfonic acid.

al., 2002); therefore, it is possible that they are a source of the ATP, which then acts on these cells in an autocrine or paracrine manner. The factors that determine the release of ATP from microglia have not been identified. Microglia possess glutamate receptors (Gottlieb and Matute, 1997) that are of the α-amino-hydroxy-5-methyl-isoxazole-4-proprionate (AMPA) receptor type (Noda et al., 2000; Bezzi et al., 2001; Hagino et al., 2004) as well as the N-methyl-D-aspartic acid (NMDA) receptor type (Gottlieb and Matute, 1997). In this study, we show that the principal synaptic excitatory transmitter, glutamate, is a powerful activator of ATP release from microglia, and we identified the mechanisms involved.

Materials and Methods

Microglial Culture. Protocols for handling animals in this study were reviewed and approved by the Animal Ethics Committee at the University of Sydney, Australia. Microglia were obtained from 1- to 2-day-old neonatal Sprague-Dawley rats provided by Laboratory Animal Services, University of Sydney (Sydney, Australia). The rats were anesthetized and killed by i.p. injection with 0.2 ml of Lethabarb (325 mg/ml pentobarbital; Virbac, Peakhurst, Australia), and the lumbar region of the spinal cords were dissected and placed in ice-cold Hank's balanced salt solution (0.4 g/l KCl, 0.06 g/l KH_2PO_4, 0.35 g/l $NaHCO_3$, 8 g/l NaCl, 0.12 g/l $Na_2HPO_4 \cdot 12H_2O$, and 1 g/l D-glucose). After carefully removing the meninges with forceps, the spinal cord segments were placed in 1 ml of 0.25% trypsin (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C for 20 min. After centrifugation, the pellet of neurons and glial cells was suspended in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% cosmic calf serum (HyClone, Logan, UT) and 1% penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA) and placed in culture flasks precoated with poly-D-lysine (20 μg/ml, Sigma-Aldrich). Culture flasks were incubated at 37°C in a 5% CO_2 atmosphere, and culture medium was exchanged with fresh supplemented DMEM every 2 to 3 days. Ten to fourteen days after initial plating, astrocytes formed a confluent monolayer on the flask surface, whereas microglia and oligodendrocytes grew loosely attached on the surface of the astrocyte monolayer and were detached by gentle shaking of the culture flask. Dead cells in the culture medium were removed and fresh medium was added. Culture flasks were then placed on a rotating shaker (IKA-VIBRAX-VXR; Janke and Kunkel, Staufen, Germany) at 200 rpm for 40 min at 37°C. Microglia that detached from the astrocyte monolayer in the medium were removed and pelleted by centrifugation. The pellet of microglia was re-suspended in the supplemented DMEM and then placed onto poly-D-lysine–coated glass coverslips in a 24-well plate. The culture medium was exchanged at 15 min because microglia selectively adhere to the poly-D-lysine coating, whereas other cell types that may be present, such as astrocytes and oligodendrocytes, take a longer period of time to adhere (Dobrenis, 1998). The coverslips were incubated with the supplemented DMEM for 2 h at 37°C to facilitate attachment of microglia. The culture medium was then replaced with Gly/Ser-free and serum-free DMEM containing 1 mg/ml bovine serum albumin (BSA; Sigma-Aldrich) which promotes microglia to be in their resting state (Tanaka et al., 1998). Induction of microglia into their activated state was achieved by addition of 0.1 μg/ml lipopolysaccharide (LPS) for 24 h (Tanaka et al., 1998). The purity of the microglial cultures was greater than 95%, which was confirmed by live staining of the cells with microglial marker FITC-IB4 (Invitrogen). Purified-microglial cultures of 2 to 3 days were used for the experiments.

Mixed glial cells, including microglia from CFTR knockout mice and from normal colony heterozygotes (control wild type) were cultured using the same method as that for culture of rat glial cells. Four-week-old female CFTR knockout UNE mice and control wild-type mice were provided by Dr. David Parsons (Women's and Chil-

dren's Hospital, Adelaide, Australia), who originally purchased the mice from The Jackson Laboratory (Bar Harbor, ME). However, the microglia were harvested using a different approach because few microglia were obtained by the shaking method used for obtaining rat microglia. Microglia were purified using 0.125% trypsin dissolved in DMEM (15-min incubation at 37°C) to remove the layer of confluent glial cells in the culture flask. The remaining un-detached glial cells (microglia) were further removed with 0.25% trypsin dissolved in Hank's balanced salt solution (5 min at 37°C). The purity of microglia was >98% confirmed by the staining with live-microglial marker FITC-IB4.

On-Line Bioluminescence Assay for ATP Measurement. ATP released into bulk solution was detected using a real-time luciferin-luciferase bioluminescence assay (Liu et al., 2005) using a luminometer (P30CWAD5-45 Photodetector Package; Electron Tubes, Ruislip, UK). In brief, excess luciferin-luciferase (1 mg/ml ATP assay mix; Sigma-Aldrich) was added to cultured microglia bathed with HEPES buffer (140 mM NaCl, 5 mM KCl; 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4). Photon counts were high initially, and gradually reached a steady value after 30 to 60 min. Stable ATP release for 10 min was treated as baseline. To examine the effect of each chemical on ATP release, the chemicals at 10% of original volume on the coverslip were added using a micropipette without washout until the end of the experiments. Each chemical tested and each solvent used to dissolve the chemicals in this experiment, such as dimethyl sulfoxide, were added using a micropipette to the ATP mix in the absence of microglia to test whether the chemical was contaminated with ATP and whether it affected the luciferin-luciferase activity. Neither glutamate nor its receptor subtype agonists, including NMDA, AMPA, kainate, and 1S,3R-1-amino-cyclopentane-1,3-dicarboxylic acid (ACPD) were contaminated with ATP. The final concentration of dimethyl sulfoxide, methanol, acetone, and chloroform used to dissolve chemicals was less than 0.1%, and these solvents had no effect on ATP release from microglia or on luciferin-luciferase activity. For those experiments using pertussis toxin (Sigma-Aldrich), botulinum toxin A (Calbiochem, San Diego, CA), and tetanus toxin (Sigma-Aldrich), microglia were preincubated with the toxins in the culture medium for 24 h. To maximize the blocking effect on G-proteins, microglia were preincubated with GDPβS (Sigma-Aldrich) for 3 to 16 h. Microglia were incubated with the other modulators in HEPES buffer for at least 40 min before application of glutamate. ATP released from the cells was determined from the photon counts using a standard ATP–photon count curve. Only a single dose of glutamate was added to microglia on each coverslip in this study and was not washed out during experimentation.

The number of cells on the coverslips used for the bioluminescence experiments was initially checked with a light microscope and finally determined by application of 1% Triton X-100 at the end of experiments to release all intracellular ATP. The total amount of ATP reflects the number of cells on the coverslips. If the total ATP level differed by >10% from the average, the result was discarded. Fewer than 10% of all coverslips fell into this category.

Immunohistochemistry. Immunohistochemistry was carried out to confirm both culture purity and the presence of glutamate receptors on microglial cells. Detailed protocol for the immunohistochemistry was described previously (Liu et al., 2005). Purified microglial cultures were stained with mouse anti-rat CD11b monoclonal antibody (Chemicon, Temecula, CA), a microglial marker to differentiate microglia from macroglia including astrocytes and oligodendrocytes that were identified using monoclonal glial fibrillary acidic protein (GFAP) antibody (Sigma-Aldrich) and galactocerebroside (Chemicon), respectively. Rabbit anti-rat polyclonal antibodies were used to identify the expression of glutamate receptor subunits in the microglia. These antibodies were anti-NR1 (NMDA receptors; Chemicon), anti-gluR2/3 (Sigma-Aldrich), and anti-gluR4 (AMPA receptor subunits; Tocris, Bristol, UK), and anti-mGluR1α/5 (Chemicon) (metabotropic glutamate receptor subtype; Sigma-Aldrich). All

the primary antibodies above were used at a 1:100 dilution except 1:800 for GFAP. Glial cell markers (anti-CD11b, anti-GFAP, or anti-galactocerebroside) were incubated together with antibodies for glutamate receptor subunits. Polyclonal antibodies that were used to identify glutamate receptor subunits were further labeled with Alexa Fluor (AF) 594 conjugated goat anti-rabbit antibody (Invitrogen). The monoclonal antibodies that were used to identify cell types were then labeled with AF488 conjugated goat anti-mouse antibody (Invitrogen). Because the anti-galactocerebroside antibody nonspecifically labeled all cell types, the oligodendrocytes could only be identified from their morphology and lack of staining of anti-CD11b and anti-GFAP antibodies. FITC-IB4 antibody used for live labeling of microglia was performed according to the methods described by Petersen and Dailey (2004). The cells were viewed under an Axiovert 200M confocal microscope (Zeiss, Jena, Germany), and images were acquired with a digital camera (AxioCam; Zeiss).

Patch-Clamp Recording. Whole-cell currents were recorded in the microglial cells, which were positively labeled with the live-microglial marker FITC-IB4 at room temperature (22–24°C) using Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA). The currents were recorded under voltage clamp mode at a holding membrane potential of −60 mV. The pipette resistance was 4 to 8 MΩ using a patch pipette solution (145 mM CsCl, 1 mM MgCl$_2$, 10 mM EGTA, and 10 mM HEPES, pH 7.3); the external bath solution was 140 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 10 mM HEPES, pH 7.4. Magnesium was omitted from the bath solution when recording NMDA currents. The currents were sampled on-line using a Digidata 1322A interface and pClamp8 program (Molecular Devices). Glutamate and its analogs were applied through pressure ejection via a picospritzer (General Valve, Fairfield, NJ).

Ca^{2+} Imaging. The level of intracellular free Ca^{2+} in microglia was measured using Fluo4-AM with a Zeiss microscope (Axiovert 200M; Zeiss). Cells grown on coverslips with 50 to 70% confluence were incubated in culture medium containing 4 μM Fluo4-AM (Invitrogen) at 37°C for 45 min. The coverslips were then placed in a chamber perfused with HEPES buffer at a speed of 2 ml/min. To wash out the excessive Fluo4-AM, the cells were superfused for 30 min before taking images. After control images were taken (before addition of glutamate or its agonists), the cells were superfused with 0.5 mM glutamate or its agonists for 3 min. For the experiments in the presence of receptor antagonist or an intracellular Ca^{2+} store release inhibitor or inositol monophosphatase inhibitor, the cells were incubated with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 100 μM), thapsigargin (1 μM), or LiCl (1 mM) for 30 min before addition of glutamate. Intensity of fluorescence was viewed under the Zeiss microscope and captured with a digital camera (AxioCam) and Axiovision program (Zeiss). Images were taken every 10 s and analyzed using ImageJ software (http://rsb.info.nih.gov/ij/). Results were presented as relative fluorescence values (F/F_0), where F_0 stands for the fluorescence of controls (before addition of glutamate).

SPQ Fluorescence Assay of Functional CFTR. Changes in fluorescence intensity of SPQ was measured to assay CFTR expression according to the study by Illsley and Verkman (1987). By overnight incubation, microglia were loaded with SPQ fluorescent dye (10 mM) dissolved in culture medium. The coverslips with SPQ-loaded microglia cells were transferred to the recording chamber, which was mounted on the stage of the Zeiss microscope (Axiovert 200M; Zeiss). The cells were continually perfused with the HEPES buffer at 2 ml/min. The dye was excited at 360 nm and emitted at >435 nm. To reduce possible damage due to UV (360 nm) exposure, we set each exposure time <100 ms using an AxioCam camera (Zeiss) and Axiovision program (version 3.1; Zeiss). The image was captured every 20 s, and total images taken were <100. The images were analyzed with ImageJ, and the results presented under the *Results* section were corrected according to slopes obtained when microglia were exposed to either Cl$^-$ buffer (137 mM NaCl, 2 mM KCl.7, 0.7 mM CaCl$_2$, 1.1 mM MgCl$_2$, 1.5 mM KH$_2$PO$_4$, and 8.1 mM Na$_2$HPO$_4$, pH 7.4) or NO$_3^-$ buffer [137 mM NaNO$_3$, 2.7 mM KNO$_3$,

0.7 mM Ca(NO$_3$)$_2$, 1.1 mM Mg(NO$_3$)$_2$, 1.5 mM KH$_2$PO$_4$, and 8.1 mM Na$_2$HPO$_4$, pH 7.4]. Results were presented as relative fluorescence values (F/F_0), where F_0 stands for the fluorescence of controls (before addition of glutamate or change of buffer).

Chemicals and Statistics. Generic chemicals were purchased from Sigma-Aldrich. All experiments were repeated at least three times, and values of peak amplitudes are presented as mean ± S.E.M. Statistical significance was determined with the use of unpaired t tests, where $p < 0.05$ was considered significant.

Results

Glutamate Induced ATP Release from Cultured Spinal Cord Microglia. Glutamate-induced ATP release from microglia was initially studied in resting microglia. This resting state was induced by culturing microglia with a Gly/Ser-free and serum-free DMEM containing 1 mg/ml BSA (Tanaka et al., 1998). Resting microglia possessed few processes and glutamate (0.5 mM) induced a severalfold increase of peak ATP release above the basal ATP level (fold increase: 3.93 ± 0.31; $n = 9$; Fig. 1A). Likewise, glutamate at 0.5 mM also induced ATP release from the activated microglia prepared by incubation of the microglia with 0.1 μg/ml LPS for 24 h (3.06 ± 0.42; $n = 4$; Fig. 1B). The activated microglia appeared to have a rounder morphology with higher phase contrast (Fig. 1B). The amplitude of glutamate-stimulated ATP release from activated microglia was not significantly different from that of resting microglia. Glutamate-stimulated ATP release from microglia in either state was not due to mechanical disturbance, in that the addition of buffer to the culture did not release ATP (Fig. 1, A and B).

Because there was no statistical difference in ATP release from the two states of microglia, glutamate-stimulated ATP release from the resting microglia was used for studies on the mechanisms of ATP release. The dose-response curve possessed an ED$_{50}$ of 0.39 mM for glutamate-stimulated ATP release from resting microglia ($n = 3$ to 7; Fig. 1C). Glutamate at 0.5 mM was then used in the rest of the study. Glutamate-stimulated ATP release from 99% pure resting microglia (3.95 ± 0.6; $n = 3$) did not significantly differ from that of 95% pure microglia; therefore, microglial cultures of 95% purity were used for subsequent experiments.

AMPA Receptors Mediated Glutamate Effects. Glutamate receptor subtypes that mediated ATP release were next investigated. This was first studied using on-line bioluminescence to detect ATP release. The glutamate (0.5 mM)-stimulated ATP release was mimicked by AMPA (0.5 mM), which had a bigger effect than glutamate, although this difference was not significant (6.1 ± 1.1; $n = 6$; Fig. 2, A and B). ATP release was not induced by other glutamate subtype receptor agonists (Fig. 2B). These included NMDA (0.5 mM; 1.0 ± 0.03; $n = 4$), kainate (0.5 mM; 1.1 ± 0.04; $n = 5$), and metabotropic glutamate receptor agonist ACPD (0.5 mM; 1.2 ± 0.15; $n = 4$). Antagonist studies revealed only the AMPA/kainate receptor antagonist CNQX (100 μM; 1.7 ± 0.08; $n = 7$; $p < 0.001$) and specific AMPA receptor antagonist GYKI 52466 (23 μM; 1.2 ± 0.09; $n = 5$; $p < 0.001$) significantly decreased glutamate-stimulated ATP release (Fig. 2, A and C). Glutamate-stimulated ATP release was not significantly decreased by other glutamate receptor subtype antagonists, including NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (4.1 ± 0.68; $n = 4$) and the metabotropic glutamate receptor antagonist (S)-α-methyl-4-

carboxyphenylglycine (3.9 ± 0.56; $n = 3$; Fig. 2C). To further confirm the involvement of the AMPA receptor in the effect of glutamate, cyclothiazide (CTZ) and 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluorophenoxyacetamide (PEPA), flip and flop allosteric AMPA receptor modulators, respectively, were used to inhibit desensitization of the AMPA receptor. Glutamate-stimulated ATP release was significantly increased by PEPA (9.0 ± 0.83; $n = 5$; $p < 0.001$) but not by CTZ (3.7 ± 0.32; $n = 7$; Fig. 2B).

To further confirm the identity of the functional glutamate receptors on microglial cell membranes that mediated glutamate-stimulated ATP release, we recorded whole-cell membrane currents. Glutamate (20 of 44 cells) and AMPA (6 of 16 cells) induced inward currents (Fig. 2D). Kainate (one of eight cells) and NMDA (two of eight cells) rarely induced inward currents (Fig. 2D). Among the inward currents in-

duced by glutamate, three were fast transient currents (<30 ms to peak and <100 ms to recover with peak amplitudes ranging between 11 and 250 pA). The rest of the glutamate-induced inward currents were slow currents (>100 ms to peak and >1000 ms to recover) with peak amplitudes ranging between 20 and 30 pA. AMPA-induced currents were also slow currents, and amplitudes ranged between 25 and 90 pA. This result supports experiments suggesting that that glutamate-stimulated ATP release occurs mainly through the AMPA receptors on the microglial cell membrane.

Because the results of the bioluminescence studies indicated that AMPA receptors were responsible for glutamate-stimulated ATP release, and glutamate receptor currents were recorded from the microglia, the expression of glutamate receptor subtypes was then confirmed using immunohistochemistry techniques. AMPA receptor subunits gluR2/3 and gluR4, NMDA receptor subunit NR1, and metabotropic
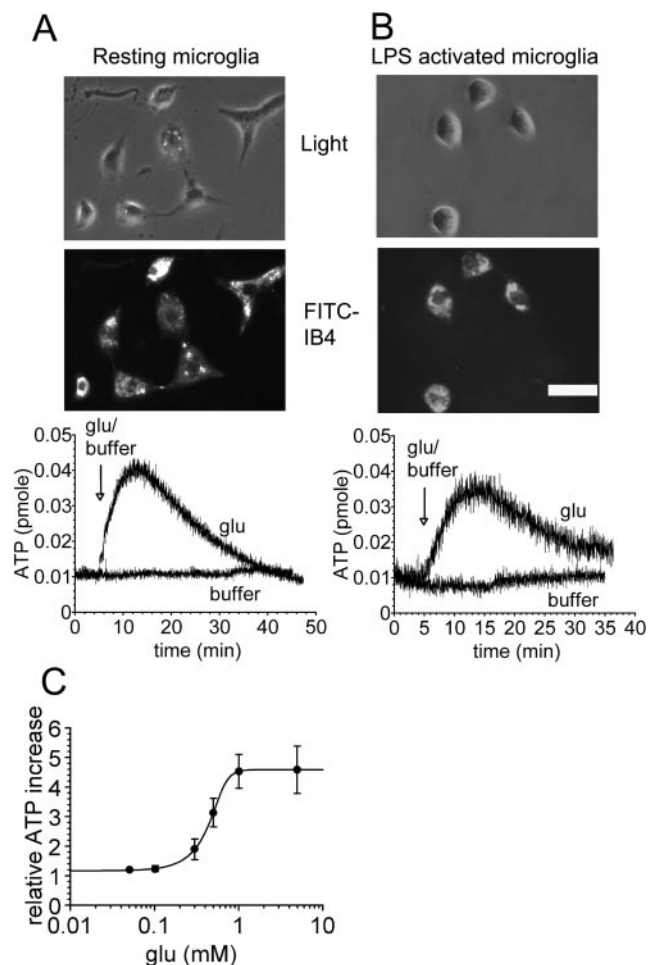

Fig. 1. Glutamate (glu) induced ATP release from different functional states of microglia cultured from rat spinal cord. A, resting microglia achieved by culturing the cells in Gly/Ser-free and serum-free culture media released ATP stimulated by 0.5 mM glutamate. The top two images are the microglia taken under light and fluorescence of FITC-IB4 antibody, a marker for live microglia. The bottom line graph shows the time course of ATP release after application of glutamate or after application of physiological buffer as a control to determine whether there was mechanical disturbance by application of glutamate. B, activated microglia induced by incubation with 0.1 μg/ml LPS for 24 h also released ATP stimulated by glutamate in the same manner as that of resting microglia. The upper images show the activated microglia and lower line graph shows the time course of ATP release. C, dose-response curve of glutamate-stimulated ATP release from resting microglia (without LPS treatment). The white scale bar in B represents 20 μm and holds for A and B.
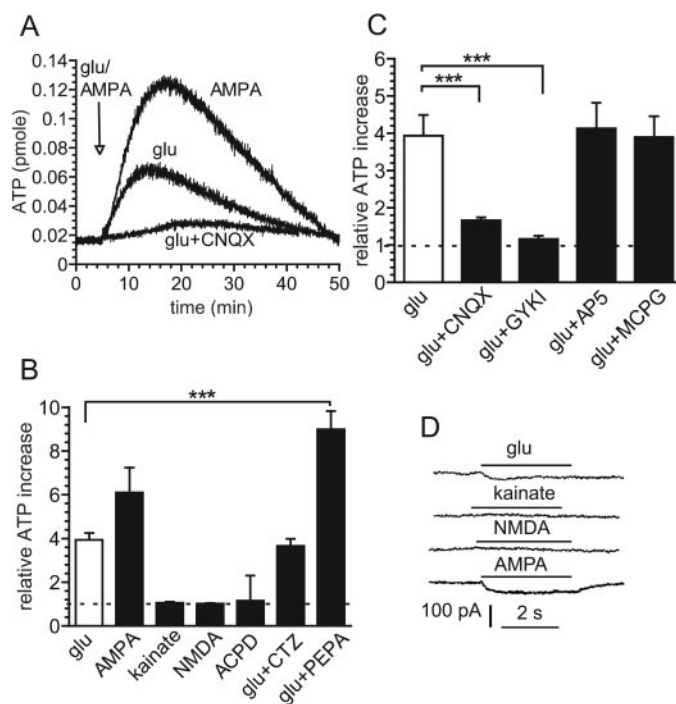

Fig. 2. AMPA receptors mediate glutamate-stimulated ATP release from microglia. A, time course of glutamate (0.5 mM)-stimulated and of AMPA (0.5 mM)-stimulated ATP release. The glutamate response is inhibited by the AMPA/kainate receptor antagonist CNQX (100 μM). B, histogram of summarized effects of glutamate agonist-stimulated ATP release from microglia. AMPA at 0.5 mM induced ATP release with a larger amplitude than that of glutamate, whereas other agonists NMDA (0.5 mM), kainate (0.5 mM), and ACPD (0.5 mM, a metabotropic glutamate receptor agonist) did not induce significant ATP secretion from the microglia. The glutamate-stimulated ATP release was significantly enhanced by PEPA (100 μM), a flop-preferring allosteric modulator of AMPA receptors but was not altered by CTZ (100 μM), a flip-preferring allosteric modulator of AMPA receptors. C, histogram of glutamate receptor antagonists on glutamate-stimulate ATP release from the microglia. Both the AMPA/kainate receptor antagonist CNQX (100 μM) and specific AMPA receptor antagonist GYKI 52466 (GYKI, 23 μM) significantly inhibited glutamate-stimulated ATP release, whereas NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (100 μM) and metabotropic glutamate receptor antagonist (S)-α-methyl-4-carboxyphenylglycine (100 μM) failed to alter glutamate-stimulated ATP release. Dashed lines in B and C and the following figures indicate the level of basal ATP release (before application of glutamate or its agonists). D, glutamate and AMPA at 0.5 mM for both compounds induced whole-cell membrane currents recorded from FITC-IB4 labeled microglia but no currents were produced on application of NMDA or kainate at 0.5 mM. ∗∗∗, $p < 0.001$.

glutamate receptor mGluR1α/5 were positively expressed on cultured microglia (Fig. 3). The glutamate receptor subunit with strongest staining was the gluR2/3 subunit of the AMPA receptor. Although NMDA and metabotropic glutamate receptors were not involved in glutamate-stimulated ATP release from the microglia, they were positively expressed in microglia (Fig. 3).

Activation of a PTX-Insensitive G-Protein and Protein Kinase C and Ca^{2+} Release from Intracellular Ca^{2+} Stores Were Necessary for Glutamate-Stimulated ATP Release. Ionotropic glutamate receptors, but not gluR2 subunits, are channels in the cell membrane that allow the influx of extracellular Ca^{2+} upon activation. The functions of ionotropic glutamate receptors are usually dependent on the rise in intracellular Ca^{2+} concentration incurred by Ca^{2+} influx. Because the glutamate-induced ATP release observed in this study involved the ionotropic AMPA receptor, the dependence of the response on the influx of extracellular Ca^{2+} was tested. Glutamate-stimulated (0.5 mM) ATP release from the microglia was not significantly affected in Ca^{2+}-free HEPES buffer solution containing the Ca^{2+} chelator EGTA (5 mM; 4.1 ± 0.36; $n = 3$; Fig. 4B). This indicates that AMPA receptor-induced ATP release does not require the influx of extracellular Ca^{2+}. Thus, we then examined whether the release of Ca^{2+} from intracellular stores was necessary for ATP release. Glutamate-induced ATP release was significantly reduced after incubating microglia with 1 μM thapsigargin, which inhibits the release of Ca^{2+} from intracellular compartments (1.2 ± 0.04; $n = 7$; $p < 0.001$; Fig. 4, A and B). Because thapsigargin may be toxic to cells, microglia were treated for 1 h with 1 μM thapsigargin epoxide, the inactive form of thapsigargin. Thapsigargin epoxide

did not significantly affect glutamate-induced ATP release (3.4 ± 0.46; $n = 3$; Fig. 4B). Thus the inhibition of ATP release in the presence of thapsigargin was not a result of toxicity. These data indicate that glutamate-induced ATP release requires mobilization of Ca^{2+} from intracellular stores, whereas influx of extracellular Ca^{2+} is not involved.

To investigate the glutamate-induced increase in intracellular free Ca^{2+} through the release from intracellular Ca^{2+} stores, we then used Ca^{2+}-imaging to monitor the intracellular free Ca^{2+} of microglia loaded with Fluo4-AM. Glutamate (0.5 mM) triggered Ca^{2+} increases that were mimicked by AMPA (0.1 and 0.5 mM) and inhibited by AMPA/kainate receptor antagonist CNQX (100 μM) and thapsigargin (1 μM; Fig. 4C) but unaffected by removal of extracellular Ca^{2+} (glutamate applied in Ca^{2+} free buffer; Fig. 4C). These results support those examining the mechanisms of glutamate-stimulated ATP release.

Because glutamate-stimulated ATP release was dependent not on Ca^{2+} influx but on Ca^{2+} release from intracellular stores, it would suggest that a G-protein coupled metabotropic signaling pathway is involved in glutamate-stimulated ATP release from microglia. Glutamate-stimulated ATP release was significantly decreased by a 3- to 16-h preincubation in 200 μM GDPβS, a nonhydrolyzable GDP analog to inhibit G-proteins ($1.3. \pm 0.15$; $n = 6$; $p < 0.001$; Fig. 5B). The
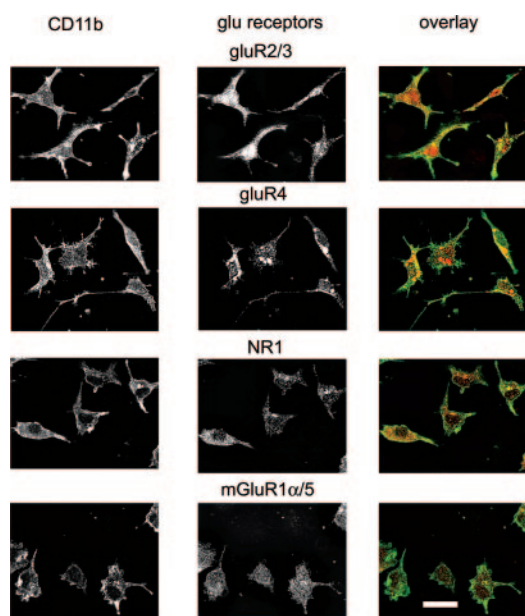

Fig. 3. Expression of glutamate receptor subtypes in microglia. Glutamate receptor subtypes AMPA, NMDA, and metabotropic glutamate receptor positively stained with gluR2/3 and gluR4 antibody for AMPA receptors, NR1 antibody for NMDA receptor, and mGluR1α/5 antibody for metabotropic glutamate receptors. The images in the left column show microglia stained with microglial marker CD11b antibody, and the images in the middle column show microglia stained with antibodies for glutamate receptor subtypes. The images in the right column are the overlay of microglia staining with antibodies for CD11b (green) and glutamate receptor subtypes (red). The white scale bar represents 20 μm.
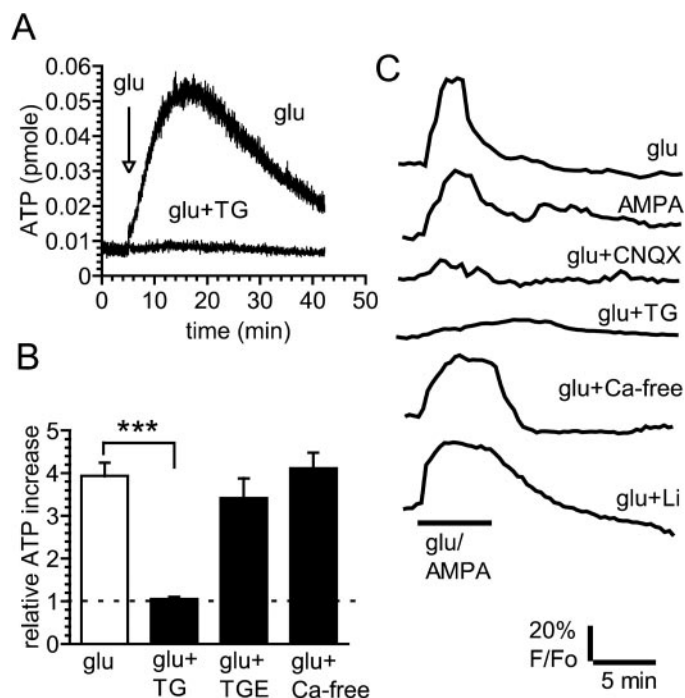

Fig. 4. Ca^{2+} release from intracellular Ca^{2+} stores is necessary for glutamate-stimulated ATP release from microglia. A, time course of glutamate-stimulated ATP release from microglia that was blocked by 1 μM thapsigargin (TG, Ca^{2+}-ATPase inhibitor). B, histogram shows that glutamate-stimulated ATP release was significantly attenuated by depletion of intracellular Ca^{2+} with thapsigargin (TG) but not significantly changed by removal of extracellular Ca^{2+}. The inactive form of thapsigargin, thapsigargin epoxide (1 μM, TGE), did not effect glutamate-stimulated ATP release. ***, $p < 0.001$. C, Ca^{2+}-imaging shows that glutamate-induced Ca^{2+} transients were mimicked by AMPA (0.5 mM) but were attenuated by AMPA/kainate receptor antagonist CNQX (100 μM) and thapsigargin (1 μM). Glutamate-induced Ca^{2+} transients were not decreased either by removal of extracellular Ca^{2+} or by Li$^+$ (1 mM), but their peak times were longer than other traces. Each trace in C was the average of at least six cells from three different coverslips.

PTX sensitivity of the involved G-protein was examined by PTX preincubation for 24 h. Glutamate-stimulated ATP release was affected by neither 100 ng/ml (4.2 ± 0.81; $n = 5$; Fig. 5B) nor 2 μg/ml PTX (4.0 ± 0.48; $n = 4$). It was then explored whether activation of other metabotropic receptors could also induce ATP release using UTP, which stimulated ATP release from astrocytes (Abdipranoto et al., 2003) and Schwann cells (Liu et al., 2005) through the metabotropic P2Y2 receptor. UTP (10 μM) induced ATP release from the cultured microglia (5.2 ± 0.15; $n = 4$), and this release was abolished by the purine receptor antagonist 100 μM suramin.

The involvement of protein kinase C (PKC), an intracellular signaling molecule, in glutamate-stimulated ATP release was also investigated. PKC inhibitor chelerythrine chloride significantly inhibited glutamate-stimulated ATP release (20 μM; 1.4 ± 0.06; $n = 7$; $p < 0.001$; Fig. 5, A and B) and
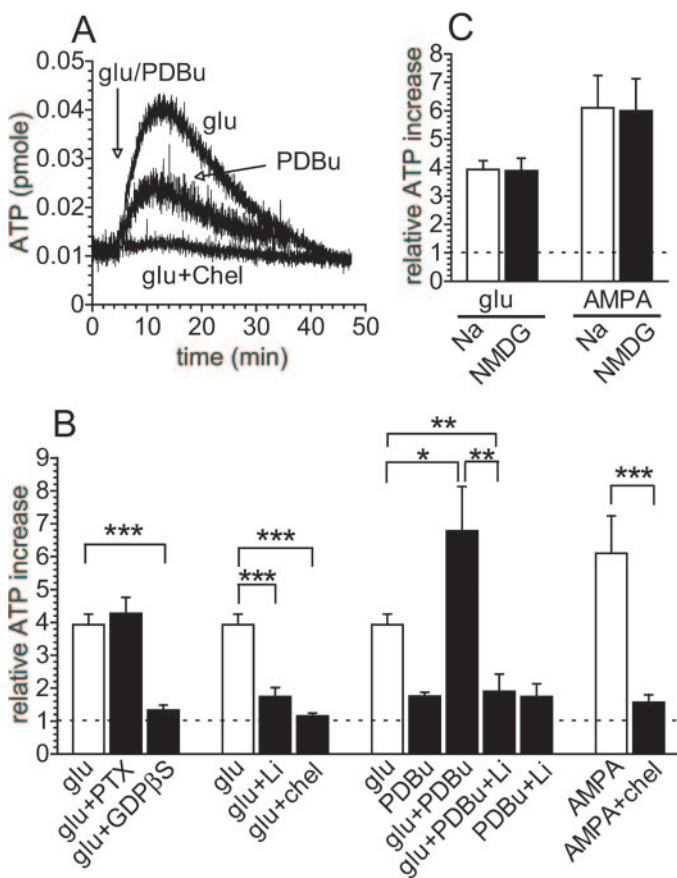

Fig. 5. Intracellular pathways that mediate glutamate-stimulated ATP release from microglia. A, glutamate-stimulated ATP release from microglia and PKC activator PDBu (1 μM)-stimulated release of ATP. Glutamate-stimulated ATP release was attenuated by PKC inhibitor chelerythrine (20 μM, Chel). B, histogram of statistical results of experiments examining the role of G-proteins and the PKC pathway in glutamate-stimulated ATP release. Glutamate-stimulated ATP release was significantly decreased by G-protein inhibitor GDPβS (200 μM) but was not affected by PTX (100 ng/ml). PKC inhibitor chelerythrine (20 μM) significantly attenuated glutamate-stimulated and AMPA (0.5 mM)-stimulated ATP release. PDBu (1 μM) significantly enhanced the effect of glutamate. Inositol monophosphatase inhibitor LiCl (Li, 1 mM) significantly decreased glutamate-stimulated and glutamate (1 mM) + PDBu (1 μM)-induced ATP release, but did not significantly affect PDBu (1 μM)-induced ATP release. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. C, glutamate- or AMPA-stimulated ATP release from the microglia was not affected by replacement of Na⁺ with NMDG⁺ in HEPES buffer solution to prevent glutamate-induced membrane depolarization on the opening of the AMPA receptors.

AMPA-stimulated ATP release (1.6 ± 0.22; $n = 5$; $p < 0.001$; Fig. 5B), indicating that PKC activation was also necessary for glutamate-induced ATP release. The involvement of the PKC pathway was confirmed by using phorbol 12,13-dibutyrate (PDBu; 1 μM), which mimicked the glutamate effect if it was applied alone (1.8 ± 0.11; $n = 11$; Fig. 5, A and B). Glutamate-stimulated ATP release was significantly enhanced by coapplication of PDBu and glutamate (8.25 ± 0.25; $n = 3$; $p < 0.001$; Fig. 5B). We also investigated whether activation of intracellular pathways involving PKC and Ca^{2+} occurred as a result of membrane depolarization after activation of AMPA receptors. NaCl in the HEPES buffer solution was substituted with NMDG-Cl, which does not pass through the opened AMPA receptor channel, so the membrane does not depolarize. A small amount of NaCl (1.4 mM) remained in NMDG-Cl-HEPES buffer solution as a component of the original ATP assay mix. Glutamate- (3.9 ± 0.44; $n = 6$) and AMPA- (0.5 mM; 6.0 ± 1.1; $n = 5$) stimulated ATP release from microglia in NMDG-Cl-HEPES buffer solution did not significantly differ from that in NaCl-HEPES buffer solution (Fig. 5C). These data suggest that stimulation of microglia with glutamate led to G-protein activation, Ca^{2+} release from intracellular stores, and activation of PKC as part of the pathway leading to ATP release. LiCl also significantly decreased glutamate-stimulated ATP release (1 mM; 1.7 ± 0.28; $n = 8$; $p < 0.001$; Fig. 5B) and glutamate plus PDBu-stimulated ATP release from cultured microglia (1.9 ± 0.53; $n = 5$; $p < 0.01$; Fig. 5B). However, Li^+ could not alter glutamate-induced intracellular Ca^{2+} transients (Fig. 4C) and did not significantly affect PKC activator PDBu (1 μM)-induced ATP release (1.7 ± 0.18; $n = 11$; Fig. 5B). This suggests that Li^+ affects the signaling pathway for glutamate-stimulated ATP release at a step downstream of phospholipase C and Ca^{2+} mobilization

Membrane ABC Transporter CFTR Was Involved in ATP Release Mechanisms. To investigate the mechanism of ATP secretion, the four main ATP secretion mechanisms, including exocytosis, efflux through connexin hemichannels, and transport via the CFTR and anion transporters on microglial cell membranes, were tested for their involvement in microglial glutamate-induced ATP release. Glutamate-stimulated ATP release from microglia was significantly inhibited by the CFTR inhibitors glibenclamide (100 μM; 2.6 ± 0.23; $n = 8$; $p < 0.01$; Fig. 6, A and B) and flufenamic acid (100 μM; 1.8 ± 0.35; $n = 4$; $p < 0.01$; Fig. 6B).

To examine expression of functional CFTR on microglia, an SPQ fluorescence assay was used. Fluorescence of cells is quenched by an increased intracellular Cl^- concentration (Illsley and Verkman, 1987). Fluorescence intensity of SPQ in microglia perfused with either Cl^- buffer or NO_3^- buffer was decreased after UV exposure because of dye leakage, and this decrease in fluorescence was well fitted by two exponentials ($n = 43$). The slope was used to correct the rest of the results. All experiments were performed 5 min after the coverslips were transferred to the recording chamber. Glutamate (0.5 mM) reversibly increased the fluorescence intensity by 10% in the NO_3^- buffer ($n = 35$; Fig. 6C). In the presence of glutamate (0.5 mM), the intensity of fluorescence in microglia was decreased 30% by switching buffers from NO_3^- to Cl^- and back to NO_3^- ($n = 35$; Fig. 6C). These glutamate-induced changes were greater than those in the

absence of glutamate ($n = 26$; Fig. 6C). This decrease almost recovered after reperfusion with NO_3^- buffer.

The CFTR-mediated, glutamate-stimulated ATP release from microglia was further examined using CFTR knockout UNE mice and colony normal heterozygotes (control wild-type mice). Glutamate (0.5 mM)-stimulated ATP release from microglia of control wild-type mice had a similar time course (Fig. 6D) but a greater amplitude (5.2 ± 0.46; $n = 5$; Fig. 6E) compared with that from rat microglia. Glutamate-stimulated from microglia of CFTR knockout mice was significantly lower (2.1 ± 0.22; $n = 6$; $p < 0.001$) than that from the control (Fig. 6, D and E).

The hemichannel inhibitor 18β-glycyrrhetinic acid (30 μM) did not alter glutamate-stimulated ATP release in this study
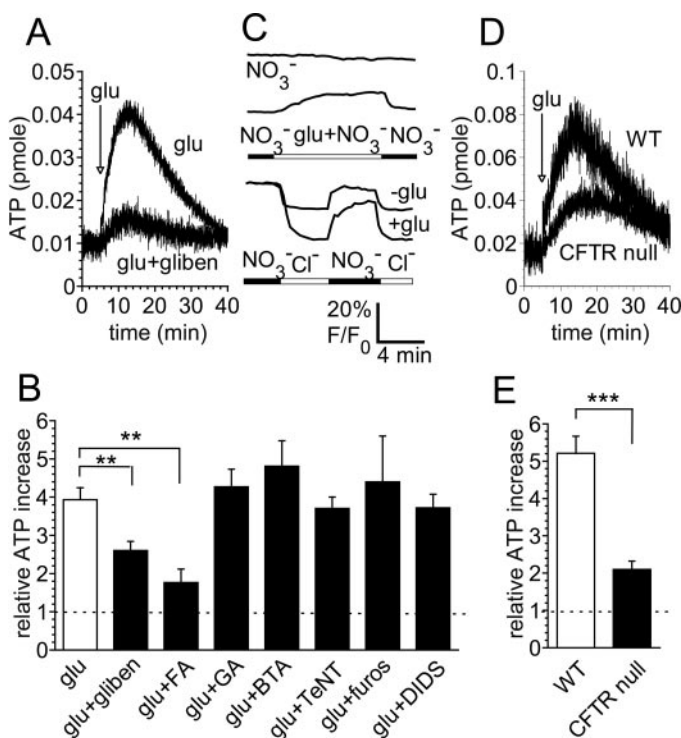

Fig. 6. Glutamate-stimulated ATP release involves the CFTR on the cell membrane. A, time course of glutamate-stimulated ATP release, which was attenuated by CFTR inhibitor glibenclamide (100 μM, gliben). B, histogram that summarizes mechanisms of glutamate-stimulated ATP release from microglia. CFTR inhibitors glibenclamide (100 μM) and flufenamic acid (100 μM, FA) significantly decreased glutamate-stimulated ATP release from the microglia. The hemichannel inhibitor 18β-glycyrrhetinic acid (100 μM, GA) did not change the glutamate effect. The glutamate-stimulated ATP release was not significantly affected by exocytosis inhibitor botulinum toxin A (15 nM and 24 h incubation, BTA) and by tetanus toxin (300 ng/ml, 24-h incubation, TeNT). The glutamate-stimulated ATP release was not affected by either chloride channel blocker DIDS (100 μM) or the anion transporter furosemide (1 mM, furos). ∗∗, $p < 0.01$. C, an SPQ fluorescence assay revealed existence of functional CFTR in microglia. The upper trace is the control (average) in which microglia were in NO_3^- bath solution with UV excitation (<100 ms exposure and 20 s interval). The middle trace shows that glutamate (0.5 mM) reversibly increased fluorescence intensity in NO_3^- solution. The lower trace shows that in the absence (−glu) and presence of glutamate (0.5 mM, +glu) fluorescence intensity was reversibly decreased by switching to Cl^- solution and recovered (partly recovered with glutamate) after switching back to NO_3^- solution. Glutamate induced a greater change of fluorescence intensity than the control (−glu). D, time course of glutamate-stimulated ATP release from microglia of CFTR knockout (CFTR-null) and control wild type (WT) mice. E, histogram shows that glutamate-stimulated ATP release from microglia of CFTR knockout mice was significantly less than that from microglia of control wild type.

(4.3 ± 0.46; $n = 3$; Fig. 6B). Treatment of cells with 15 nM botulinum toxin A for 24 h (to cleave syntaxin) to inhibit exocytosis did not alter the morphology of microglia and did not significantly affect glutamate-stimulated ATP release (4.8 ± 0.66; $n = 6$; Fig. 6B). Likewise, preincubation of microglia with tetanus toxin (to cleave synaptobrevin, 300 ng/ml) for 24 h to inhibit exocytosis did not decrease glutamate-stimulated ATP release (3.7 ± 0.29; $n = 6$; Fig. 6B). However, increasing the concentration of tetanus toxin to 2 μg/ml significantly enhanced glutamate-stimulated ATP release (5.76 ± 0.81; $n = 5$; $p < 0.01$). Glutamate-stimulated ATP release was not significantly affected by the chloride channel antagonist diisothiocyanstilbene disulfonic acid (DIDS; 100 μM; 3.7 ± 0.36; $n = 3$) or by the anion transporter inhibitor furosemide (1 mM; 4.4 ± 1.7; $n = 3$; Fig. 6B). These results suggest that glutamate-stimulated ATP release from microglia occurs through ATP efflux via CFTR.

Discussion

In the present study, we have shown that glutamate-stimulated ATP release from spinal cord microglia occurred through the activation of AMPA receptors, which were antagonized by the specific AMPA receptor antagonist GYKI 52466 and AMPA/kainate receptor antagonist CNQX. Activation of AMPA receptor-induced ATP release was independent of Ca^{2+} influx and cell membrane depolarization because removal of extracellular Ca^{2+} and substitution of extracellular Na^+ with $NMDG^+$ did not affect glutamate-stimulated ATP release. However, the release occurred via a Ca^{2+}-dependent PKC pathway that was antagonized by lowering Ca^{2+} in the endoplasmic reticulum with thapsigargin and the PKC inhibitor chelerythrine. Intracellular signaling pathways involved in effects of AMPA receptor activation have been documented previously. AMPA receptors could modulate G-proteins to directly activate voltage-gated channels in central neurons (Kawai and Sterling, 1999; Satake et al., 2004; Takago et al., 2005). Glutamate-stimulated ATP release from microglia was inhibited by G-protein inhibitor GDPβS and was insensitive to PTX, indicating that G_q (not G_i) G-proteins were involved in the signaling pathway. In this study, we have also demonstrated that UTP stimulated ATP release from microglia. UTP evoked a G-protein-dependent current in microglia (Norenberg et al., 1997) and induced Ca^{2+} mobilization and prolactin release through G_q proteins in pituitary lactotrophs (He et al., 2003), as well as stimulating ATP release from the microglia. In addition, AMPA receptor activation of kinase pathways in the cerebellum is independent of the influx of Ca^{2+} (Krieger et al., 2000).

Antagonists for NMDA and metabotropic glutamate receptors did not alter the extent of glutamate-evoked ATP release, whereas those for the AMPA receptor did. Furthermore, agonists to the NMDA and metabotropic glutamate receptors did not produce significant levels of ATP release, whereas those to the AMPA receptor (but not kainate) did. Taken together, the results point to AMPA receptor activation being uniquely involved in glutamate-stimulated ATP release from spinal cord microglia. This is also supported by the fact that under patch-clamp only, AMPA-evoked currents were recorded, but not those to NMDA or kainate. This points to a lack of functional high-affinity kainate receptors on these

spinal-cord microglia, as has been reported for cortical microglia (Hagino et al., 2004), although kainate at a concentration of 0.5 mM scarcely induced currents presumably as a consequence of binding to the low-affinity binding site for kainate on the AMPA receptors. NMDA receptor subtype NR1 was observed with immunohistochemistry, together with the gluR2/3 and gluR4 AMPA receptor subtypes that are known to exist on these cells (Noda et al., 2000). We have no explanation for our failure to observe constant NMDA or kainate-induced currents, although neither kainate nor NMDA gave significant release of ATP.

Spinal cord microglia studied here seem to have quite distinct responses to kainate and AMPA compared with cortical microglia. Only small currents were recorded in spinal cord microglia to kainate, although substantial currents were generated by AMPA. The opposite is the case for cortical microglia (Noda et al., 2000). Furthermore, whereas CTZ greatly potentiates the current due to AMPA in cortical microglia, it had no significant effect on glutamate-evoked ATP release from spinal cord microglia. PEPA and CTZ inhibited glutamate-induced tumor necrosis factor-α release from cortical microglia (Hagino et al., 2004). However, PEPA but not CTZ significantly increased the glutamate-evoked release of ATP from spinal cord microglia.

We have not distinguished between the capacity of gluR1–gluR4 subunits of AMPA receptors to evoke ATP release. The gluR2 receptor is dominant in determining the ionic conductance of hetero-oligomers, and in the absence of this subunit, the receptors show a significant Ca^{2+} conductance (Swanson et al., 1997). Our Ca^{2+} imaging of microglia exposed to glutamate or AMPA shows that the intracellular Ca^{2+} increases to approximately the same extent over several minutes in each case, and this was almost completely antagonized by CNQX and thapsigargin but was not affected by removal of extracellular Ca^{2+}. The fact that thapsigargin produces a very significant decrease in glutamate-evoked ATP release and Ca^{2+}-free solutions failed to affect the glutamate effect is consistent with the source of most of the rise in intracellular Ca^{2+} coming from the endoplasmic reticulum for activation of the Ca^{2+}-dependent PKC. Glutamate-stimulated ATP release was inhibited by the inositol monophosphatase inhibitor Li^+. Activation of phosphatidylinositol-phospholipase C leads to generation of inositol trisphosphate and triggers Ca^{2+} mobilization. However, Li^+ did not inhibit Ca^{2+} transients induced by glutamate. This suggests that Li^+ inhibits the signaling pathway for ATP release at step(s) downstream of Ca^{2+} mobilization. Li^+ is known to interfere with the translocation of PKC to membranes in some cell types, so inhibiting PKC activation (Wang et al., 2001). This action probably explains the over 2-fold decrease in the action of glutamate in releasing ATP in the presence of Li^+ and the 4-fold decrease in the action of glutamate and PDBu in releasing ATP in the presence of Li^+. The relatively small effect of PDBu in releasing ATP compared with that of PDBu together with that of glutamate is probably due to glutamate releasing Ca^{2+} for the Ca^{2+}-dependent lipid binding domain of PKC as well as activating the diacylglycerol/phorbol ester site, thus promoting the maximum efficiency of PKC (Quest and Bell, 1994).

Given our evidence that there is functional CFTR expression in microglia revealed by SPQ fluorescence assay, and that CFTR modulates ATP secretion on activation of AMPA receptors with glutamate, the question arises as to whether PKC activates the CFTR. There is ample evidence that PKC phosphorylates the regulatory subunit of CFTR, leading to its activation (see, for example, Duan et al., 2005). The CFTR possesses a regulatory domain with approximately 20 potential sites for phosphorylation by protein kinases (Picciotto et al., 1992; Bompadre et al., 2005). Both Ca^{2+}-independent and -dependent isoforms of PKC activate the CFTR (Berger et al., 1993). This channel is blocked by flufenamic acid (McCarty et al., 1993) and by glibenclamide (Schultz et al., 1996). Because each of these drugs produced a significant decrease in glutamate-stimulated ATP release, it is likely that the CFTR is a principal means of regulating glutamate-evoked ATP release. Flufenamic acid also blocks secretion of ATP through C38, C43, C46, and C50 connexins (Stout et al., 2002; Bahima et al., 2006), but these are also blocked by 18β-glycyrrhetinic acid (Ye et al., 2003), which does not affect glutamate-stimulated ATP release from microglia. A variety of chloride channels are also blocked by flufenamic acid (Greenwood and Large, 1995), but only one of these, a voltage-gated channel, has been reported as also blocked by glibenclamide at high concentrations (Meyer and Korbmacher, 1996). However, this channel is also blocked by DIDS, which has no effect on glutamate-evoked ATP release from microglia, so that it is unlikely that these chloride channels are involved in glutamate-evoked ATP release. It was further confirmed that the CFTR is involved in the mechanisms of glutamate-stimulated ATP release from microglia by the finding that glutamate-stimulated ATP release from microglia of CFTR knockout mice is significantly less than that from microglia of control wild-type mice.

There is considerable literature claiming that the CFTR is not involved in ATP release (see, for example, Grygorczyk and Hanrahan, 1997; Mitchell et al., 1998), that it is involved in ATP release (see, for example, Prat et al., 1996; Cantiello, 2001), and that such release involves interaction between the CFTR and a separate ATP channel (see, for example, Jiang et al., 1998; Braunstein et al., 2001). It seems likely at present that in those cells in which ATP release is modulated by CFTR activity, the ATP release mechanism is not an inherent part of the CFTR, as is its chloride channel, but rather linked to the CFTR by PDZ (postsynaptic density-95, discs large, zone occludens-1) domains as is the case with other channels (for a review, see Li and Naren, 2005).

Exocytosis is not involved in glutamate-stimulated ATP release from cultured spinal cord microglia. Both botulinum toxin A and tetanus toxin did not affect glutamate-stimulated ATP release. This finding differs from that of glutamate-stimulated ATP release from cultured rat Schwann cells, which was via exocytosis in that it was inhibited by botulinum toxin A (Liu and Bennett, 2003). The glutamate-stimulated ATP release from microglia suggests the following two possibilities. One is that exocytotic proteins responsible for binding with botulinum toxin A and tetanus toxin do not exist in microglia. To date, there is no reported expression of exocytotic proteins in microglia. The other possibility is that exocytotic proteins exist in microglia, but exocytosis is not involved in the mechanisms of glutamate-stimulated ATP release from microglia. If this is the case, this result suggests that glutamate-stimulated ATP release occurs through different mechanisms in different cell types.

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