Activation of microglia with zymosan promotes excitatory amino acid release via volume-regulated anion channels: the role of NADPH oxidases

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Abstract

Microglia are the resident immune cells of the CNS, which are important for preserving neural tissue functions, but may also contribute to neurodegeneration. Activation of these cells in infection, inflammation, or trauma leads to the release of various toxic molecules, including reactive oxygen species (ROS) and the excitatory amino acid glutamate. In this study we used an electrophysiological approach and a D-[^{3}H]aspartate (glutamate) release assay to explore the ROS-dependent regulation of glutamate-permeable volume-regulated anion channels (VRACs). Exposure of rat microglia to hypoosmotic media stimulated Cl^- currents and D-[^{3}H]aspartate release, both of which were inhibited by the selective VRAC blocker DCPIB. Exogenously applied H_2O_2 potently increased swelling-activated glutamate release. Stimulation of microglia with zymosan triggered production of endogenous ROS and strongly enhanced glutamate release via VRAC in swollen cells. The effects of zymosan were attenuated by the ROS scavenger MnTMPyP, and by two inhibitors of NADPH oxidase (NOX) diphenyliodonium and thioridazine. However, zymosan-stimulated glutamate release was insensitive to other NOX blockers, apocynin and AEBSF. This pharmacological profile pointed to the potential involvement of apocynin-insensitive NOX4. Using RT-PCR we confirmed that NOX4 is expressed in rat microglial cells, along with NOX1 and NOX2. To check for potential involvement of phagocytic NOX2 we stimulated this isoform using protein kinase C (PKC) activator PMA, or inhibited it with the broad spectrum PKC blocker Gö6983. Both agents potently modulated endogenous ROS production by NOX2, but not VRAC activity. Taken together, these data suggest that the anion channel VRAC may contribute to microglial glutamate release, and that its activity is regulated by endogenous ROS originating from NOX4.

Keywords

microglial cells; VRAC; glutamate release; reactive oxygen species; NOX4

Introduction

Microglia are the resident immunocompetent cells of the central nervous system. In the adult brain, microglial cells are typically found in a resting state, in which they survey their local environment for any signals indicating traumatic injury, infection, or inflammation (Kreutzberg 1996; Farber and Kettenmann 2005). Upon exposure to invading pathogens,

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neuronal debris, elevated extracellular ATP levels, or pro-inflammatory cytokines and chemokines, microglia rapidly convert to an activated state. Once activated, they produce both cytotoxic and neuroprotective molecules (Kreutzberg 1996; Nakajima and Kohsaka 2001). The most prevalent microglial toxins are IL-1β, TNFα, reactive oxygen species (ROS), and reactive nitrogen species (RNS) (Nakajima and Kohsaka 2001; Block et al. 2007). Although ROS and RNS production is an intrinsic part of the CNS response to infections, trauma, neurodegeneration, or brain tumors, it frequently results in bystander neurotoxicity. ROS and RNS, including those produced by microglia, are thought to play an important role in numerous neurodegenerative processes (Nakajima and Kohsaka 2001; Halliwell 2006; Block et al. 2007). The primary source of ROS production in microglia are NADPH oxidases, multi-subunit heme-containing enzymes that catalyze the reduction of molecular oxygen to superoxide anion (O_2^-) using NADH or NADPH as a substrate (Bedard and Krause 2007).

A less recognized mediator of microglial neurotoxicity is the amino acid glutamate, which serves as the main excitatory neurotransmitter in the CNS. Several previous studies demonstrated that activated microglia release glutamate in quantities sufficient to induce neuronal death via overactivation of ionotropic glutamate receptors (Piani et al. 1991; Piani et al. 1992; Barger and Basile 2001; Takeuchi et al. 2006). Piani and co-workers suggested that glutamate, rather than ROS and/or inflammatory cytokines, is the key factor contributing to microglial toxicity (Piani et al. 1992). Two glutamate transporting pathways that have been proposed to mediate glutamate release from microglia are the cystine-glutamate antiporter and connexin hemichannels (Piani and Fontana 1994; Barger and Basile 2001; Takeuchi et al. 2006). The cystine-glutamate antiporter (system Xc^-) exchanges one intracellular glutamate for extracellular cystine, a homodimer of cysteine, which is used for the synthesis of the antioxidant glutathione (McBean 2002). This transporter is upregulated in immune cells in response to oxidative stress in order to maintain sufficient levels of glutathione, and is abundantly expressed in activated microglia (Sato et al. 2001; Qin et al. 2006). However, Takeuchi et al. (2006) recently found that connexin hemichannels, rather than the cystine-glutamate antiporter, largely mediate microglial glutamate release and excitotoxic neuronal damage in a TNFα toxicity model. Additionally, in astrocyte cultures, it has been demonstrated that glutamate release in response to hypoosmotic swelling and chemical ischemia is mediated by two anion channels: volume-regulated anion channel (VRAC) and Gd^{3+}-sensitive maxi-anion channel (Liu et al. 2006).

VRACs are ubiquitously expressed Cl^- channels whose molecular identity remains unknown (Strange et al. 1996; Nilius and Droogmans 2003; Okada 2006). In addition to inorganic anions, VRACs are permeable to several amino acids, including the excitatory amino acids glutamate and aspartate (Kimelberg et al. 1990; Banderali and Roy 1992; Jackson et al. 1994; Abdullaev et al. 2006). Although it has not been demonstrated in microglia, VRACs may contribute to pathological glutamate release, as seen in ischemia and other neurological disorders (Kimelberg 1995; Kimelberg and Mongin 1998; Mongin and Kimelberg 2005). The presence of VRACs in mammalian cells is typically determined by measuring increases in Cl^- conductance in response to cell swelling using electrophysiology. Besides sensitivity to cell volume changes, the key biophysical characteristics of VRACs include intermediate conductance, moderate outward rectification, Eisenman's type I anion permeability sequence ($SCN^- > I^- > NO_3^- > Cl^- > F^- >$ gluconate), requirement for cytosolic ATP, and time-dependent inactivation at large positive potentials (Strange et al. 1996; Okada 1997; Nilius et al. 1997).

Several studies demonstrated that microglial cell functions are strongly regulated by changes in the expression and activity of K^+, H^+, and Cl^- channels (reviewed in Eder 2005). Volume- and/or membrane stretch-sensitive Cl^- channels, resembling VRACs, were found

in cultured microglial cells and have been proposed to participate in morphological transitions, migration, proliferation, and phagocytosis in this cell type (Schlichter et al. 1996; Eder et al. 1998; Ducharme et al. 2007). Although the primary physiological role for VRACs is regulation of cellular volume, they are also activated during other cellular processes, such as apoptosis, cell motility, and proliferation (Okada et al. 2001; Lang et al. 2000; Stutzin and Hoffmann 2006). Several recent studies have found that VRACs may be activated or positively modulated by ROS, particularly H_2O_2 (Shimizu et al. 2004; Varela et al. 2004; Haskew-Layton et al. 2005). In ventricular myocytes, endogenous ROS have been demonstrated to regulate stretch-activated and swelling-activated Cl^- currents, both likely mediated by VRAC (Browe and Baumgarten 2004; Ren et al. 2008). Since microglia robustly generate ROS as part of their immunological responses, they represent an ideal cell type for studying the impact of endogenously produced ROS on VRAC activity and VRAC-mediated release of organic osmolytes. In the present work we focused on the VRAC-mediated release of the excitatory amino acids, glutamate and aspartate, because of the significant physiological and pathological impact of these neurotransmitters on neuronal signaling and viability in the brain.

Methods

Materials

4-(2-Aaminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), apocynin, dipehnyliodonium chloride (DPI), nitroblue tetrazolium chloride (NBT), thioridazine, zymosan A (*S. cerevisiae*), and H_2O_2 (30%), were purchased from Sigma-Aldrich. Gö6983, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP), and phorbol 12-myristate 13-acetate (PMA) were acquired from EMD-Calbiochem (La Jolla, CA, USA). 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]butanoic (DCPIB) acid was from Tocris Cookson (Ellisville, MO, USA).

Cell culture preparation

Microglial cells were purified from mixed glial cultures, which were prepared from the cortices of neonatal Sprague-Dawley rat according to the procedure approved by the Albany Medical College Animal Care and Use Committee. After the isolation, the cortical tissue was enzymatically dissociated using three enzymatic extractions with protease Dispase II (Sigma-Aldrich, St. Louis, MO, USA). The first extraction was discarded and DNAse I (Sigma, St. Louis, MO, USA) was added. Dissociated cortical cells were plated in T-75 flasks and grown for two-three weeks in minimal essential medium (MEM) plus 10% fetal bovine serum (FBS) and additionally supplemented with 50 units/mL penicillin and streptomycin (all cell culture reagents were from Invitrogen, Carlsbad, CA, USA) in a humidified 5% CO_2/95% air atmosphere at 37°C. Culture medium was replaced bi-weekly, and the penicillin and streptomycin were no longer added to the media after ten days of cultivation. This procedure yields a confluent mixed glial culture largely consisting of astrocytes and microglia. For purifying microglia, mixed glial cultures were shaken for approximately two to five minutes on a titer plate shaker. Floating microglial were collected, sedimented by a brief centrifugation, resuspended in Opti-MEM supplemented with 2% B-27 serum-free supplement minus antioxidants, and plated onto 18 mm^2 glass coverslips (Caroline Biological Supply Co., Burlington, NC, USA) or cell culture dishes coated with poly-ᴅ-lysine (Sigma, St. Louis, MO, USA). Cells were maintained in Opti-MEM/B-27 for at least 12 hours before being used in subsequent experiments.

Immunocytochemistry

To verify the purity our microglia cultures, we stained the isolated microglial cells for OX-42 (CD11b), a complement receptor that within the CNS is expressed solely by

microglia. Briefly, microglial cells plated onto 18 mm^2 glass coverslips were fixed in 4% paraformaldehyde for 20 minutes at room temperature (22°C). The cells were then blocked in 10% normal goat serum (Invitrogen) for 30 minutes at room temperature. Blocked cells were incubated with an OX-42 mouse anti-rat monoclonal antibody (1:500; BD Pharmingen, San Diego, CA, USA) for two hours at room temperature, washed with physiological phosphate saline, and incubated with an Alexa488-conjugated goat anti-mouse secondary antibody (1:1,000 Molecular Probes, Carlsbad, CA, USA) for 45 minutes at room temperature. After completion of immunohisto-chemical staining microglia were additionally counterstained with DAPI (100 ng/mL; Sigma) for five minutes at room temperature. Images were analyzed with an Olympus Optical Provis AX70 microscope equipped with conventional fluorescence filters (Cy2/Alexa488: 460-500 nm excitation, 510-560 nm emission; DAPI/Hoechst: 375-400 nm excitation, 450-475 nm emission). Fluorescent images were captured with a high-resolution camera (Sony, DKC-ST5) interfaced with Northern Eclipse (Empix Imaging, Mississaugau, ON), Photoshop (Adobe, San Jose, CA), and Neurolucinda (MicroBrightField, Colchester, VT) software. This procedure revealed that >98% cells were positive for OX-42 (CD11b) and therefore are of microglial origin.

Whole-cell patch clamp recordings

Microglial cells were plated on poly-D-lysine-treated glass coverslips at low density in MEM supplemented with heat-inactivated horse serum (HIHS, Invitrogen). After three hours they were transferred into B-27-supplemented Opti-MEM as described above. Adhered cells were moved into electrophysiological media described below. Whole-cell recordings were performed at room temperature as described previously (Kubo and Okada 1992; Abdullaev et al. 2006). Patch electrodes were fabricated from borosilicate glass capillaries using a micropipette puller (P-97, Sutter Instruments, Novato, CA), with a resistance of 3-3.5 MΩ when filled with pipette solution. Series resistance was ≤15 MΩ. Currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). pCLAMP software (version 9.2, Axon Instruments) was used for command pulse control, data acquisition, and analysis. Series resistance was compensated in all experiments. Current signals were filtered at 2 kHz using a four-pole Bessel filter and digitized at 4 kHz. The time course of current development was monitored by applying every 15 seconds alternating one-second step pulses from a holding potential of 0 to test pulses ±40 mV. After attaining steady-state activation of Cl$^-$ currents, their biophysical properties were determined by applying 2-s step pulses from 0 mV to test potentials of -100 to +100 mV in 20-mV increments. Short prepulse to -100 mV were applied before each test pulse to assure complete activation of VRAC channels. The isoosmotic external solution contained (in mM): 110 CsCl, 2 CaCl$_2$, 1 MgSO$_4$, 5 glucose, 10 HEPES, and 60 mannitol (pH 7.4, 290 mosM). The hypoosmotic solution was made by omitting mannitol from isotonic solution and had an osmolarity of 230 mosM. The pipette solution contained (in mM): 110 CsCl, 1 MgSO$_4$, 1 Na$_2$-ATP, 0.3 Na$_2$-GTP, 15 Na-HEPES, 10 HEPES, and 1 EGTA (pH 7.3, 255 mosM). The osmolarity of the pipette solution was set lower than that of the isotonic bath solution in order to prevent spontaneous cell swelling after attaining the whole-cell mode (Worrell et al. 1989).

Excitatory amino acid efflux assay

The release of excitatory amino acids from primary microglia was measured using a non-metabolized L-glutamate/L-aspartate analog, D-[^{3}H]aspartate, as described elsewhere (Mongin and Kimelberg 2002). In brief, primary microglia plated on 18 mm^2 glass coverslips were loaded for 3 hours with 8 μCi/mL D-[^{3}H]aspartate in MEM plus 10% HIHS. Loaded microglial cells were washed of extracellular isotope and serum-containing medium with HEPES-buffered solution, and then placed in a Lucite perfusion chamber. Cells were suffused with isoosmotic or hypoosmotic media at 1.2 mL/min. The isoosmotic medium

contained (in mM) 135 NaCl, 3.8 KCl, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 1.2 KH_2PO_4, 10 D-glucose, and 10 HEPES (pH 7.4). The hypoosmotic medium was prepared by reducing NaCl concentration to 85 mM, which translates into a 30% reduction in the medium osmolarity. Suffused media fractions were collected at one minute intervals. Isotope (D-[^{3}H]aspartate) content in each fraction was determined using a PerkinElmer Tri-Carb 1900TR liquid scintillation analyzer (PerkinElmer, Downers Grove, IL, USA) after addition of four mLs of Ecoscint A scintillation cocktail (National Diagnostics, Atlanta, GA, USA). The isotope remaining in the cells was extracted with a solution containing 2% sodium dodecylsulfate plus 8 mM EDTA. Percent fractional isotope release for each time point was determined by dividing radioactivity released in each 1-minute interval by the radioactivity remaining in the cells at this time point, as retroactively calculated using a custom computer program.

Nitroblue tetrazolium (NBT) assay

The quantitation of O_2^- production in primary microglia was performed using a nitroblue tetrazolium assay as described elsewhere (Choi et al. 2006). In brief, primary microglial cells grown on 24-well culture plates were incubated with nitroblue tetrazolium chloride and various compounds that promote and/or inhibit the production of O_2^- for 10 or 30 minutes at 37°C. Cells were then rinsed with warm PBS and fixed in 100% methanol. Reduced formazan particles, contained inside cells, were dissolved with 2M KOH in DMSO, and the absorbance was read at 620 nm on a BioTek ELx800 absorbance microplate reader (BioTek Winooski, VT, USA).

Total RNA isolation and RT-PCR

Total RNA was isolated from primary rat microglia using the RNAqueous-4PCR kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. The concentration and purity of the isolated microglial RNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). One μg of isolated RNA was then converted into cDNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), adhering to the manufacturer's instructions, in a Mastercycler thermocycler (Eppendorf, Westbury, NY, USA). One μl of the cDNA product was used for PCR amplification of the NOX1-4 and GAPDH transcripts, using rat specific primers included in Table 1.

Statistical analysis

The statistical analyses of the data were performed using either Student's t-test or one-way ANOVA followed by Tukey's post-hoc analysis for multiple comparisons. Results of the excitatory amino acid efflux assays were separately analyzed for the maximal release values under hypo-osmotic conditions, or integral release values during the whole duration of the hypoosmotic exposure. Because statistical significances of the data were very similar for both parameters, we presented only comparisons of maximal releases in the text and figures. Origin 6.0 (OriginLab, Northampton, MA) and Prism 5 (GraphPad, San Diego, CA) were used for statistical analysis.

Results

Characterization of swelling-activated, glutamate-permeable pathway in primary rat microglia

In our initial experiments, we tested for the functional expression of a volume-sensitive Cl^- permeability pathway in primary rat microglial cells using a whole-cell patch clamp electrophysiological approach. We hypothesized that microglia, as the majority of other cells, express VRACs. To activate VRACs, we induced cell swelling by exposing microglia

to hypoosmotic medium (20.7% reduction in medium osmolarity), which elicited slowly developing, outwardly rectifying Cl$^-$ currents (Fig. 1a). The average current density in five successfully patched cells was 40.8 ±5.7 pA/pF at +40 mV. This was in the same order of magnitude as the VRAC current densities registered under identical experimental conditions in primary rat astrocytes (19.7 ±3.3 pA/pF, Abdullaev et al., 2006). The registered currents were similar to those defined for VRAC in other cell types in several respects. Similar to what is shown in Fig. 1b, in all five cells, swelling-activated Cl$^-$ currents were outwardly rectifying and showed time-dependent inactivation at +100 mV, although it was less pronounced than in other cell types. Importantly, these Cl$^-$ currents were completely and reversibly inhibited by DCPIB, a pharmacological compound that discriminates VRACs from other Cl$^-$ channels (Decher et al. 2001; Abdullaev et al. 2006).

To determine whether VRACs expressed in primary microglia are permeable to glutamate, we employed an excitatory amino acid efflux assay. Primary microglia were loaded with D-[^{3}H]aspartate, a non-hydrolyzable analog of glutamate, and then suffused with isoosmotic or hypoosmotic media. The exposure of microglia to hypoosmotic medium (30% reduction in medium osmolarity) resulted in a significant release of D-[^{3}H]aspartate, and this effect was potently suppressed by 20 μM DCPIB, suggesting that microglial VRACs are permeable to glutamate (Fig. 1c).

Exogenous H$_2$O$_2$ enhances the release of glutamate from primary microglia via VRAC

It has been demonstrated in various cell types, including astrocytes, that VRACs may be activated or modulated by the reactive oxygen species hydrogen peroxide (H$_2$O$_2$) (Shimizu et al. 2004; Varela et al. 2004; Haskew-Layton et al. 2005; Ren et al. 2008). To address the question of whether H$_2$O$_2$ impacts VRAC activity in microglia, we exposed microglia to exogenous H$_2$O$_2$ under isoosmotic and hypoosmotic conditions. In non-swollen cells, 500 μM H$_2$O$_2$ produced a small gradual increase in D-[^{3}H]aspartate release levels (Fig. 2a, ~2-fold increase above basal levels, p<0.05, repeated measures ANOVA). However, when applied in conjunction with hypoosmotic medium, H$_2$O$_2$ significantly potentiated the swelling-activated D-[^{3}H]aspartate release from microglia by ~70% (Fig. 2a). The effect of H$_2$O$_2$ was completely inhibited by DCPIB, suggesting that VRAC is the source (Fig. 2a). DCPIB on its own had no antioxidant properties, as verified in ROS assays in microglia and macrophages (data not shown). We further explored which concentrations of H$_2$O$_2$ are most effective in regulating VRAC activity. We found a biphasic effect of H$_2$O$_2$ with the maximal stimulation at 100 μM (Fig. 2b). At higher H$_2$O$_2$ concentrations, potentiation of excitatory amino acid release was diminished, which may suggest a redox-dependent inactivation of VRAC itself or its regulatory mechanism(s) (Fig. 2b). To assure that the effect of H$_2$O$_2$ is mediated by increases in VRAC activity, we additionally performed several electrophysiological experiments in which H$_2$O$_2$ was applied to microglia under isoosmotic or hypoosmotic conditions. Similar to glutamate release results, 100 μM H$_2$O$_2$ did not increase basal Cl$^-$ currents in non swollen cells (n=3, data not shown). However, when 100 μM H$_2$O$_2$ was applied in conjunction with hypoosmotic medium, after swelling-activated Cl$^-$ currents reached steady-state levels, there was additional 54% increase in current density (from 45.9 ±2.6 to 70.7±3.9 pA/pF, n=3, p=0.006).

Zymosan stimulates the endogenous production of O$_2^-$ and enhances microglial VRAC activity in a ROS-dependent manner

To determine whether endogenous ROS can modulate VRAC activity, we stimulated microglia with zymosan (500 μg/mL). Zymosan is a desiccated preparation of yeast cell wall commonly employed to stimulate the activity of the ROS-producing enzyme NADPH oxidase (NOX) in immune cells (DeChatelet et al. 1975). We measured endogenous ROS production using a nitroblue tetrazolium (NBT) assay, which detects O$_2^-$ via quantification

of NBT reduction to formazan (Choi et al. 2006). Stimulation of microglia with zymosan strongly increased NBT reduction (Fig. 3a). The effect of zymosan was evident at 10 minutes, but roughly tripled after a 30-minute incubation (data not shown). In all the subsequent experiments we used a 30-minute incubation to increase assay sensitivity. Several previous studies found increased ROS production upon application of hypoosmotic media (Lambert 2003; Ortenblad et al. 2003; Varela et al. 2004). In our microglial experiments we failed to register changes in NBT reduction under hypoosmotic conditions; also, hypoosmotic medium did not increase the zymosan-induced ROS production (data not shown). The effect of zymosan was blocked by DPI (10 μM), a potent NOX inhibitor (Bedard and Krause 2007) (Fig. 3a). This suggests that zymosan stimulates the production of ROS in microglia via NOX activation.

We further tested zymosan in the excitatory amino acid release assay. Zymosan was applied for 10 minutes prior to and during the 10-min hypoosmotic stimulation. In non-swollen cells, zymosan caused a small and transient increase in D-[^{3}H]aspartate release (Fig. 3b, 2.4-fold increase above basal levels, p=0.021, marked by arrow). In swollen microglia, zymosan stimulation potentiated hypoosmotic D-[^{3}H] aspartate release by ~two-fold (Fig. 3b). As in the case of exogenous H_2O_2 (see Fig. 2a), 20 μM DCPIB suppressed zymosan-induced excitatory amino acid efflux both under isoosmotic and hypoosmotic conditions (Fig. 3b), confirming VRAC contribution. The zymosan-enhanced release of D-[^{3}H]aspartate was also blocked with 10 μM DPI, under both isoosmotic and hypoosmotic conditions (Fig. 3c), suggesting NOX involvement.

Because the effect of zymosan in non-swollen cells was very small, in all the subsequent experiments we applied zymosan during exposure to hypoosmotic medium only. Such application produced a consistently stronger stimulation of volume-sensitive excitatory amino acid release (increased from ~2-fold to ~4-fold, compare Fig. 3c to Fig. 4b).

To further confirm that the effects of zymosan on VRAC activity are mediated by ROS, rather than by activation of ROS-independent intracellular signaling pathway(s), we applied MnTMPyP, which is a cell permeable superoxide dismutase (SOD) and catalase mimetic (Day et al. 1997). One-to-30 μM MnTMPyP dose-dependently reduced NBT-detectable levels of O_2^- in microglia stimulated with zymosan (Fig. 4a). However, even at the highest MnTMPyP concentration used, inhibition of ROS production incomplete (Fig. 4a). We have chosen 20 μM MnTMPyP concentration for the subsequent experiments in order to reduce risk of toxicity and assure specificity of the effects. 20 μM MnTMPyP significantly attenuated the zymosan-enhanced release of D-[^{3}H]aspartate under hypoosmotic conditions (Fig. 4b), strongly suggesting that the effects of zymosan are mediated by ROS.

To further implicate NOX as the source of ROS involved in the regulation of VRAC activity in microglia, we employed apocynin, another commonly used inhibitor of NOX, which is more selective than DPI (Stolk et al. 1994). We used the concentration of 2 mM, because at lower concentrations apocynin showed limited efficacy in NBT assays (data not shown). Two mM apocynin decreased the zymosan-stimulated production of O_2^- in microglia by 75% (Fig. 5a) but, surprisingly, was completely ineffective against the zymosan-enhanced release of D-[^{3}H] aspartate (Fig. 5b). Similarly, AEBSF, a protease inhibitor that is frequently used to block the NOX2 isoform (Bedard and Krause 2007), prevented the zymosan-activated ROS production, but was ineffective against zymosan-stimulated glutamate release (Supplementary Fig. 1).

In an attempt to resolve the incongruity between results with DPI, apocynin and AEBSF, we tested thioridazine, another NOX inhibitor. Thioridazine is a phenothiazine derivative, which blocks dopaminergic receptors, as well as all NOX isoforms via the mechanism

similar to DPI (Serrander et al. 2007). In our experiments, thioridazine completely blocked the zymosan-stimulated production of O_2^- at 30 μM (Fig. 6a), and effectively prevented the zymosan-enhanced release of D-[^{3}H]aspartate from swollen microglia (Fig. 6b).

Expression of different NOX isoforms in microglia

Because apocynin and AEBSF were unexpectedly ineffective in attenuating the zymosan-enhanced release of D-[^{3}H]aspartate, we conducted an analysis of literature on NOX pharmacology. Five NOX isoforms (NOX1-5) are expressed in humans, while only four NOX isoforms (NOX1-4) have been identified in rat (Kawahara et al. 2007). Apocynin and AEBSF block NOX1-3, but are ineffective against NOX4, while DPI and thioridazine effectively inhibit all NOX isoforms (Bedard and Krause 2007; Serrander et al. 2007). Therefore, our data suggest that NOX4 is involved in the ROS-mediated effect of zymosan on VRAC.

To determine which NOX isoforms are expressed in primary rat microglia, we probed for the mRNAs expression of all four NOX rat isoforms (NOX1-4). Using RT-PCR, we found that primary rat microglia express high levels of NOX2 along with lower levels of NOX1 and NOX4, but not NOX3 (Fig. 7). The expression of NOX4 fits well with the pharmacological profile of zymosan-enhanced D-[^{3}H]aspartate release, suggesting that NOX4, and not NOX2, may be the source of ROS that positively regulates VRAC activity.

Activation and inhibition of NOX2 modulates O_2^- production without having any impact on microglial VRAC activity

NOX2 is considered to be the main NOX isoform expressed in microglia and other phagocytic cells; it serves as a prominent source of ROS in immunological reactions (Lambeth 2004), and, thus, is expected to be activated in response to zymosan. Our pharmacological data imply that NOX4, and *not* NOX2, is the source of ROS which impacts VRAC activity. NOX1-3, but not NOX4, require protein kinase activity for the phosphorylation-dependent assembly of an active NOX complex (Lambeth 2004; Bedard and Krause 2007). Therefore, we pharmacologically manipulated PKC activity in an attempt to discern whether ROS production by NOX2 (and perhaps NOX1, which is also expressed in rat microglia) regulates VRAC.

First, we interrupted the generation of ROS via NOX1/2 by inhibiting PKC with the broad-spectrum PKC blocker Gö6983 (Gschwendt et al. 1996). One μM Gö6983 completely blocked the zymosan-stimulated production of O_2^- (Fig. 8a) but had no effect on the zymosan-enhanced release of D-[^{3}H] aspartate (Fig. 8b).

We then took an opposite approach by stimulating PKC directly using phorbol 12-myristate 13-acetate (PMA), which has been demonstrated to fully activate NOX2, as well as NOX1 and NOX3 (Bedard and Krause 2007). 500 nM PMA was more effective in stimulating microglial O_2^- production than zymosan (Fig. 9a). However, at the same concentration, PMA failed to enhance the VRAC-mediated release of glutamate in microglia (Fig. 9b).

Together, the experiments presented in Figs. 8 and 9 strongly suggest that NOX2, the major microglial NOX isoform, and possibly NOX1, are not a critical source of ROS that regulate VRAC activity in zymosan-treated cells.

Discussion

In the present work, we found that in microglia, the immunocompetent cells of the CNS, both exogenous and endogenously produced reactive oxygen species strongly enhance excitatory amino acid release via volume-regulated anion channels (VRACs). To stimulate

endogenous ROS production by NADPH oxidases, we challenged microglial cells with zymosan, thereby mimicking pathogenic infection. The surprising finding of this study is that the major phagocytic NOX isoform, NOX2, although activated by zymosan, does not contribute to VRAC regulation. Instead, such regulation is likely mediated by NOX4, which —until the present study—has not been identified in microglial cells.

VRACs contribute to glutamate release from microglia

Microglial activation in response to pathogenic infection or trauma is important for the preservation of neural tissue (Kreutzberg 1996; Nakajima and Kohsaka 2001; Block and Hong 2005). When activated, microglia produce and release various inflammatory cytokines, reactive oxygen and nitrogen species, and glutamate (Nakajima and Kohsaka 2001; Block and Hong 2005; Block et al. 2007). Glutamate is the primary excitatory neurotransmitter in the CNS, which may induce excitotoxic tissue damage if its extracellular levels are not properly regulated (Choi 1988; Choi 1992). One of several potential pathways for glutamate release are VRACs (Kimelberg and Mongin 1998; Mongin and Kimelberg 2005; Malarkey and Parpura 2008). In microglial cells, swelling-activated anion channels, which resemble VRAC, have been identified and suggested to play important roles in volume regulation, cell proliferation, and phagocytosis (Schlichter et al. 1996; Ducharme et al. 2007). Importantly, microglial swelling-activated Cl$^-$ channels are permeable to glutamate and aspartate with permeability values, relative to Cl$^-$, of ~0.18 and ~0.22, respectively (Ducharme et al. 2007). However, Ducharme et al. (2007) have found that microglial swelling-activated anion channels are not inactivated at positive potentials and have much smaller unitary conductance of ~1-3 pS in comparison to a prototypical VRAC. Conversely, our electrophysiological and pharmacological data suggest that rat microglia express classical VRACs. Several characteristics that support such a notion include: (i) outward rectification of the swelling induced Cl$^-$ currents, (ii) time-dependent current inactivation at positive potentials, and (iii) the sensitivity of swelling-activated Cl$^-$ currents to the selective VRAC blocker DCPIB. DCPIB has been found to discriminate VRACs from other cloned to-date Cl$^-$ channels (Decher et al. 2001). Additional studies are needed to determine if the method of cell preparation or differences in recording solutions explain variation between present electrophysiological findings and those in the work of Schlichter and co-workers (Ducharme et al. 2007). Along with our electrophysiological data, the inhibitory effect of DCPIB on amino acid release suggests that microglial VRACs serve as a major route for glutamate release, and in such a way may profoundly impact neural tissue function. This may especially be the case under pathological conditions associated with cell swelling or in the processes of microglial migration, proliferation, and phagocytosis, which likely involve enhanced VRAC activity (Schlichter et al. 1996; Eder 2005; Ducharme et al. 2007).

Microglial VRACs are potently regulated by exogenous and endogenous ROS

The major objective of this study was to determine if ROS may trigger glutamate release from microglial cells via glutamate-permeable VRACs. In several cell types, it has been demonstrated that ROS activate or positively modulate VRACs (Browe and Baumgarten 2004; Shimizu et al. 2004; Varela et al. 2004; Haskew-Layton et al. 2005; Ren et al. 2008), as well as similar volume-sensitive taurine permeability pathway (Ortenblad et al. 2003; Lambert 2003). Because activated microglia abundantly generate ROS, these cells represent an ideal subject for studying redox regulation of VRACs, and the functional impact of such regulation on cell physiology and function. In our experiments, application of exogenous H$_2$O$_2$ enhanced hypoosmotic medium-induced D-[^{3}H]aspartate release and VRAC Cl$^-$ currents via a DCPIB-sensitive pathway, acting synergistically with cell swelling. Consistent with our previous findings in astrocytes (Haskew-Layton et al. 2005), H$_2$O$_2$ was essentially ineffective in non-swollen cells. Induction of endogenous ROS production with zymosan

produced limited VRAC activation in non-swollen cells, and potently increased glutamate release by 2-5-fold from microglial cells exposed to hypoosmotic media. These data suggest that ROS preferentially modulate VRACs only when these channels are already active. The magnitude of D-[^{3}H]aspartate release from non-swollen cells exposed to H_2O_2 or zymosan is too small to have physiological significance. Under isoosmotic conditions set in our experiments, VRACs are inactive. Therefore, we subject microglial cells to large hypotonic stimuli in order to activate the volume-sensitive Cl^- currents and glutamate fluxes. Such hypoosmotic gradients do not exist in the brain, even in pathology. However, microglial VRACs may be activated under various physiological and pathological conditions without changes in extracellular osmolarity. Such a notion is supported by the observations that nonselective VRAC blockers inhibit proliferation, phagocytosis and shape transitions in microglia (Schlichter et al. 1996; Eder et al. 1998; Ducharme et al. 2007).

Zymosan is commonly used to stimulate ROS production in immune cells, including microglia, and this effect is mediated via activation of NADPH oxidases (Colton and Gilbert 1987; Sankarapandi et al. 1998). In our experiments, the effects of zymosan on D-[^{3}H]aspartate release via VRACs were inhibited either by the antioxidant MnTMPyP or the NOX blockers DPI and thioridazine, which strongly suggests that they are mediated by ROS originating form NOX.

Differential impact of NOX isoforms on regulation of microglial VRAC by ROS

The paradoxical finding of the present work was insensitivity of zymosan-induced VRAC activation to the NOX inhibitors apocynin and AEBSF. Apocynin, DPI, AEBSF, and thioridazine *all* potently suppressed zymosan-induced superoxide production. However, only DPI and thioridazine inhibited VRAC-mediated glutamate release from zymosan-treated cells. This discrepancy may be explained by suggesting that the effect of zymosan on VRAC involves atypical NOX4. NOX1-3 isoforms require phosphorylation-dependent assembly of several cytosolic subunits in order to be catalytically active, while NOX4 activity does not require the same proteins and is regulated via alternative mechanisms (Bedard and Krause 2007, see Fig. 10). Apocynin and AEBSF suppress the activity of NOX2 (and related NOX1 and 3) by inhibiting the assembly of regulatory subunits (Stolk et al. 1994; Bedard and Krause 2007). However, these compounds are ineffective inhibitors of NOX4 (Serrander et al. 2007). DPI and thioridazine, on the other hand, inhibit all NOX isoforms, including NOX4, by interrupting electron flow in the catalytic subunit (Bedard and Krause 2007; Serrander et al. 2007). Although DPI and thioridazine are not specific for NOXs, and also block other electron-transferring enzymes, they are the only potent NOX4 inhibitors available to-date.

NOX4 is a recently discovered enzyme, whose expression in microglial cells has not been reported until the present study (Bedard and Krause 2007). Taken together, the presence of NOX4 messenger RNA and our pharmacological data strongly support the hypothesis of NOX4 involvement in regulation of VRAC. A similar idea has been previously put forth by Lambert and colleagues, who suggest that NOX4 is critical for activation of swelling-sensitive taurine release pathway in mouse NIH 3T3 fibroblasts (Friis et al. 2008).

It should be noted, however, that NOX4 is not a major NOX isoform in microglia, nor is it known to be activated by zymosan. In microglia, zymosan stimulates generation of ROS via activation of phagocytic NOX2 (Sankarapandi et al. 1998). It was not clear to us why NOX2-derived ROS were not contributing to VRAC activation by zymosan. In order to address this question, we stimulated NOX2 by treating microglial cells with PKC activator PMA, thereby promoting the assembly of catalytically active NOX2 complex (Bedard and Krause 2007). PMA potently enhanced generation of O_2^-, but it did not affect the VRAC activity. Conversely, the potent PKC inhibitor Gö6983 completely suppressed O_2^-

generation in zymosan-treated cells, but did not affect VRAC activity. These data unequivocally prove that NOX2-derived ROS are not critical for VRAC regulation by zymosan under our experimental conditions.

The surprising lack of effect of NOX2-derived ROS may be explained by the location of ROS production and/or by the peculiarity of NOX4 biochemistry. As shown in Fig. 10, NOX2 is predominantly present in plasmalemmal and phagocytic membranes and secretes superoxide to the extracellular space or into the phagosome (Bedard and Krause 2007). In contrast, NOX4 is thought to be predominantly associated with the endoplasmic reticulum and/or endosomes and generates disproportionally higher amounts of intracellular H_2O_2 compared to other NOXs (Martyn et al. 2006;Bedard and Krause 2007;Serrander et al. 2007). Therefore, NOX4 may create sufficiently high local levels of H_2O_2 that stimulate VRACs. Our attempts to quantify intracellular H_2O_2 production in zymosan-treated cells using a fluorescent probe 5-chloromethyl-2′7′-dichlorofluorescein diacetate (CM-H_2DCFDA) were unsuccessful because of the turbidity of zymosan solutions.

Pathological significance VRAC-mediated glutamate release from microglial

Microglia activation is a common feature of many neurological disorders. Although necessary for preservation of neural tissue, microglial responses may have deleterious effects, which are largely related to the excessive production of ROS (Kreutzberg 1996; Nakajima and Kohsaka 2001; Block and Hong 2005). Genetic deletion of the ROS-producing enzyme NOX2 or its regulatory subunits, or overexpression of ROS-scavenging enzymes, are protective in animal models of cerebral ischemia, Alzheimer's and Parkinson's diseases, and methamphetamine neurotoxicity (Yang et al. 1994; Walder et al. 1997; Deng and Cadet 2000; Zhang et al. 2004; Park et al. 2005). Tissue damage in the same animal models can also be prevented by antagonists of the NMDA subtype of glutamate receptors (Sonsalla et al. 1989; Zeevalk et al. 1994; Lipton 1999). Therefore, oxidative stress and glutamate toxicity are likely related processes. It is well known how glutamate promotes oxidative and nitrosative stress (Beckman and Koppenol 1996; Lipton 1999). Whether the opposite is true, *i.e.*, if ROS production may lead to enhanced glutamate release, is less clear. Previous studies implicated the cystine/glutamate antiporter and connexin hemichannels in the ROS- and inflammation-induced glutamate release (Piani and Fontana 1994; Barger and Basile 2001; Takeuchi et al. 2006; Fogal et al. 2007). Our present and published work suggests that, in microglia, astrocytes and possibly other types of neural cells, oxidative stress may promote pathological glutamate release via the glutamate-permeable anion channel VRAC, and perhaps other permeability pathways (Haskew-Layton et al. 2005; Liu et al. 2006). Although our present *in vitro* data point to a key role for NOX4, in intact tissue NOX2 may be as important. A recent report by Z. Ren *et al.* (2008) implicated NOX2 in regulation of VRAC activity by angiotensin II in cardiac myocytes. NOX2-derived superoxide is converted extracellularly and intracellularly to H_2O_2, which in intact tissue is capable of accumulating in pathological quantities, regardless of the initial source (Halliwell 2006). In our experimental paradigm, NOX2-generated ROS may be consumed in phagosome or removed with perfusion, while NOX4-derived H_2O_2 may reach high intracellular levels (see Fig. 10). Overall, our study proposes one possible mechanism that links microglial ROS production to elevated glutamate release, which involves the anion channel VRAC. Such release may contribute to glutamate-associated neuronal damage seen in various models of neural pathologies.

Acknowledgments

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Abbreviations used

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AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (NOX2 inhibitor)
DCPIB	4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]butanoic acid (selective VRAC blocker)
DPI	dipehnyliodonium chloride (NOX inhibitor)
MEM	minimal essential medium
FBS	fetal bovine serum
NOX	NADPH oxidase
PKC	protein kinase C
ROS	reactive oxygen species
VRAC	volume-regulated anion channel
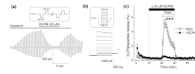
Fig. 1. Cultured primary rat microglial cells express volume-regulated anion channels, which are permeable to excitatory amino acids

(a) Representative electrophysiological recording of Cl⁻ currents in a single cultured microglial cell exposed to hypoosmotic medium. Cells were held at 0 mV and alternative one-second pulses to ±40 mV were applied to measure activation of Cl⁻ currents every 15 seconds. The VRAC blocker DCPIB (20 µM) was applied after Cl⁻ currents reached steady-state level. (b) Cl⁻ current responses to step pulses from -100 to +100 mV in 20 mV increments applied after swelling-activated Cl⁻ currents reached steady-state level. Representative traces of electrophysiological recordings in 5 cells. (c) Effect of the VRAC blocker DCPIB (20 µM) on hypoosmotic medium-induced D-[³H]aspartate release from microglia. Cells were loaded for 3 hours with D-[³H]aspartate. Extracellular isotope was then washed and cells were placed in a Lucite perfusion chamber. Data represent the mean values ±SE of six experiments. *** p < 0.001, DCPIB vs. control hypoosmotic medium (Hypo).
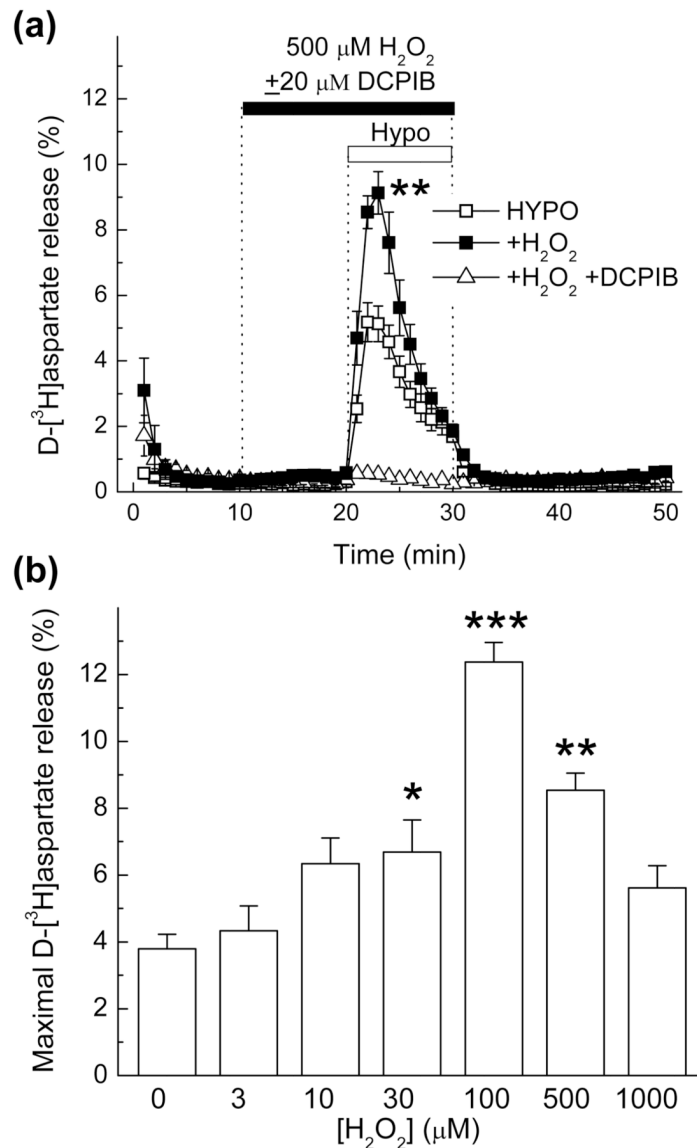
Fig. 2. Exogenous reactive oxygen species H$_2$O$_2$ potentiate the excitatory amino acid release from primary microglia via VRAC

(a) Primary microglia were exposed to H$_2$O$_2$ (500 μM) 10 minutes prior to and during the 10-minute suffusion with hypoosmotic medium in the presence or absence of the VRAC blocker DCPIB (20 μM). Data represent the mean values ±SE of six experiments. **p<0.01, H$_2$O$_2$ vs. Hypo, and p<0.001, H$_2$O$_2$ vs. H$_2$O$_2$+DCPIB. **(b)** The effect of varying concentrations of H$_2$O$_2$ on the release of D-[^{3}H]aspartate from microglia under hypoosmotic conditions. These experiments were conducted in a similar fashion as in (a). For clarity only maximal D-[^{3}H]aspartate release data are shown. Mean values ±SE of five to ten experiments in each group are presented. *p<0.05, **p<0.01, ***p<0.001, H$_2$O$_2$ vs. hypoosmotic control; one-way ANOVA with Tukey's post-hoc analysis.
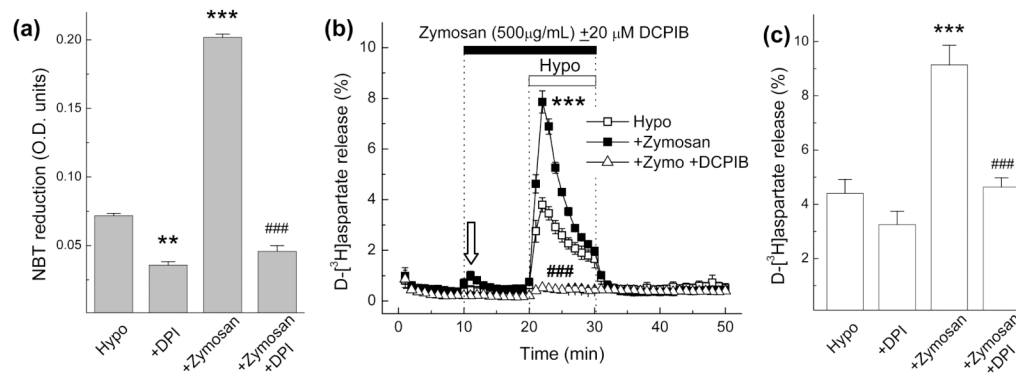
Fig. 3. Zymosan stimulates the endogenous production of superoxide anion (O$_2^-$) and enhances microglial excitatory amino acid release

(a) Effects of zymosan and the NOX inhibitor DPI on microglial production of O$_2^-$. Primary microglial cells were exposed to hypoosmotic media additionally containing zymosan (500 μg/mL) and/or DPI (10 μM) for 30 minutes at 37°C. O$_2^-$ production was measured as the reduction of NBT (see Materials and methods for details). ***p<0.001 Zymosan vs. Control; ###p<0.001 Zymosan vs. Zymosan+DPI. (b) Effect of zymosan on microglial D-[³H]aspartate release. Microglia were exposed to zymosan (500 μg/mL) for 10 minutes prior to and during 10-minute application of hypoosmotic medium. **Arrow** indicates a transient increase in D-[³H]aspartate release upon zymosan application under isoosmotic conditions. In a separate set of experiments, zymosan was applied in combination with 20 μM DCPIB. The mean values ±SE of six experiments are presented. ***p<0.001 Hypo vs. Zymosan. ###p<0.001 Zymosan+DCPIB vs. Hypo or Zymosan alone. (c) Effects of zymosan (500 μg/mL) on hypoosmotic medium-induced D-[³H]aspartate release in the presence or absence of DPI (10 μM). The design of these experiments was similar to those presented in (b); DPI was present 30 min before and during application of hypoosmotic medium. For clarity only maximal hypoosmotic release values are presented. Data are the mean values ±SE of 5-6 experiments. ***p<0.001, Zymosan vs. Hypo; ###p<0.001, Zymosan vs. Zymosan + DPI.
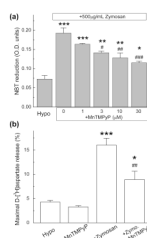
Fig. 4. Endogenous ROS production mediates zymosan effect on microglial excitatory amino acid release via VRAC

(a) The effect of the ROS scavenger MnTMPyP on intracellular O_2^- levels in microglia. Primary microglial cells were exposed for 30 minutes to hypoosmotic media containing zymosan (500 µg/mL) and various concentrations of MnTMPyP. O_2^- production was measured as the reduction of NBT. Representative of two experiments. Data are the mean values ±SE of three independent measurements. *$p<0.05$, **$p<0.01$, $p<0.001$ vs. Hypo; #$p<0.05$, $p<$Zymosan+MnTMPyP vs. Zymosan control. (b) The effect of MnTMPyP (20 µM) on the zymosan-induced enhancement of hypoosmotic D-[^{3}H]aspartate release. The mean values ± SE of five experiments are shown. **$p<0.01$, ***$p<0.001$ vs. Hypo; ##$p<0.01$, Zymo.+MnTMPyP vs. Zymosan. In these experiments one-way ANOVA with Tukey's post-hoc analysis was performed after logarithmic transformation of the data.
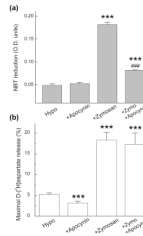
Fig. 5. The NOX blocker apocynin potently suppresses the zymosan-induced ROS production, but not the zymosan effect on VRAC activity
(a) The effects of apocynin on zymosan-induced O_2^- production in microglia. Microglial cells were exposed to hypoosmotic media additionally containing zymosan (500 μg/mL) and/or apocynin (2 mM). Apocynin was included in the media 30 minutes before and during 30-minute incubation with zymosan and NBT. Data are the mean values ±SE of four experiments. ***p<0.001, vs. Hypo; ###p<0.001 Zymosan vs. Zymo. +Apocynin. **(b)** The effect of zymosan on hypoosmotic medium-induced D-[3H]aspartate release in the presence or absence of 2 mM apocynin. The mean values ±SE of five experiments are shown. ***p<0.001 vs. Hypo. In these experiments one-way ANOVA with Tukey's post-hoc analysis was performed after logarithmic transformation of the data.
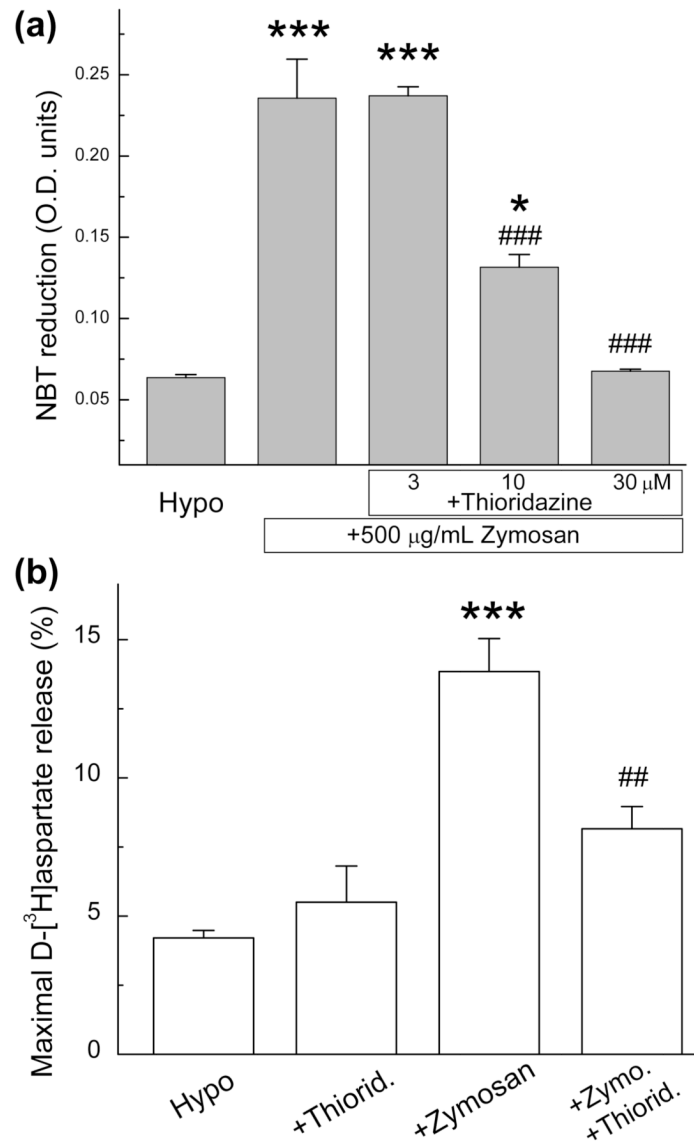
Fig. 6. The NOX inhibitor thioridazine blocks zymosan-enhanced ROS production and excitatory amino acid release

(a) The effect of various concentration of thioridazine on zymosan-induced production of O_2^- in microglia exposed to hypoosmotic medium. Thioridazine was applied for 30 minutes in conjunction with 500 μg/mL zymosan and NBT. The mean values ±SE of three independent measurements in a representative experiment are shown. *p<0.05, ***p<0.001, vs. Hypo, ###p<0.001 vs. Zymosan control. **(b)** The effect of 20 μM thioridazine on zymosan-induced enhancement of hypoosmotic D-[3H]aspartate release. The data are the mean values ±SE of five experiments. ***p<0.001 vs. Hypo. ##p<0.01 Zymozan vs. Zymo. +Thioridazine.
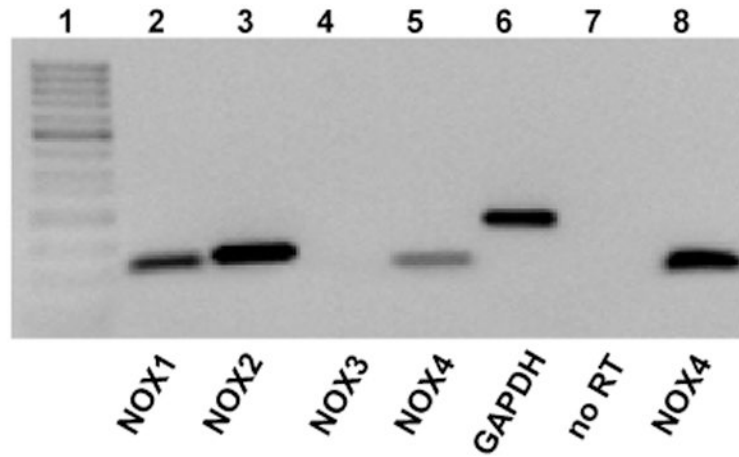
Fig. 7. NOX1, NOX2, and NOX4 isoforms of NAD(P)H oxidase are expressed in primary microglial cells

This figure shows a representative negative image of ethidium bromide staining of PCR products for transcripts of various NOX isoforms. PCR was performed after reverse transcriptase (RT) processing of the total RNA samples isolated from primary rat microglia. Total RNA was treated with DNAse in order to remove genomic DNA contamination. Lanes 2-6 show RT-PCR products for rat NOX1-4 and GAPDH in microglial samples, as indicated. NOX3 was not expressed in microglia. In the reaction presented in lane 7, PCR was performed using NOX4 primers, but the RT product was omitted. Lane 8 shows NOX4 RT-PCR product from primary rat endothelial cells (positive control). Lane 1 contains 50-bp MW ladder.
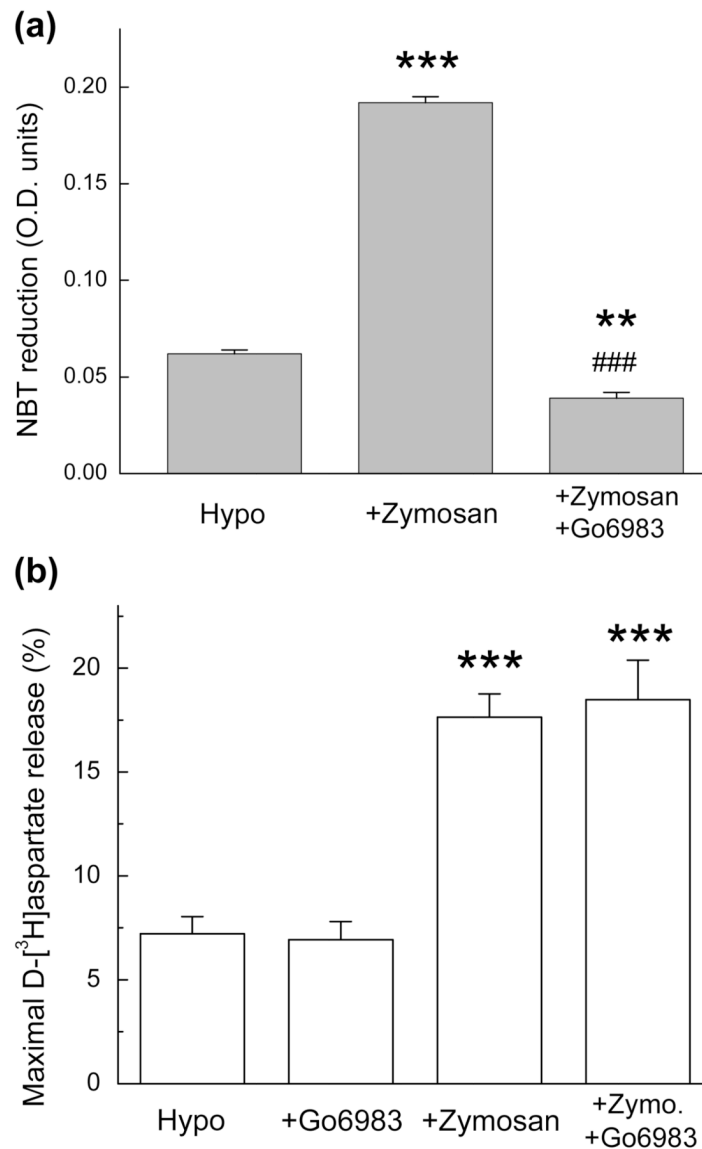
(a)

(b)

Fig. 8. The PKC inhibitor Gö6983 completely prevents NOX2-medited O_2^- production but not the zymosan-enhanced release of excitatory amino acids via VRAC
(a) Effect of Gö6983 on the zymosan-induced of O_2^- production (reduction of NBT). Microglial cells were exposed for 30 minutes to hypoosmotic medium additionally containing zymosan (500 μg/mL) and the broad-spectrum PKC inhibitor Gö6983 (1 μM). Data are the mean values ±SE of three independent measurements in a representative of three experiments. **p<0.01, ***p<0.001 vs. Hypo; ###p<0.001, Zymosan vs Zymosan +Gö6983. (b) The effect of zymosan on hypoosmotic D-[3H]aspartate release in the presence or absence of 1 μM Gö6983. Data are the mean values ±SE of six to nine experiments. ***p<0.001,. vs. Hypo.
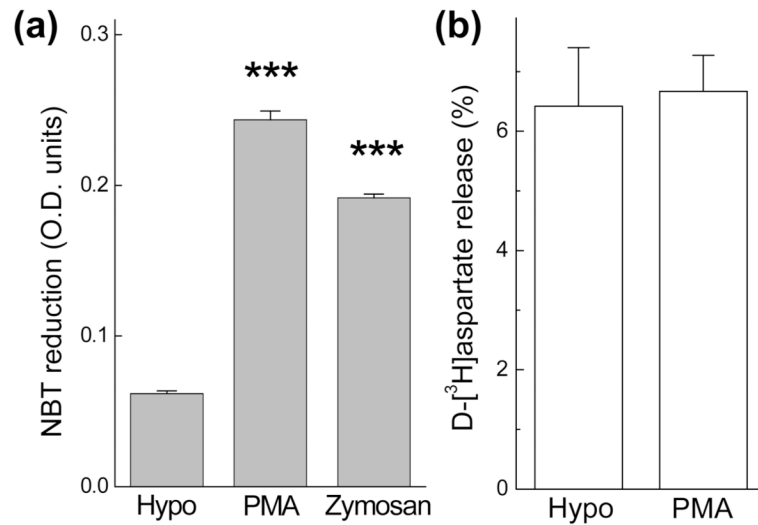
Fig. 9. PKC-dependent stimulation of NOX2 enhances O_2^- production without impacting the VRAC-mediated release of excitatory amino acids
(a) Effects of PMA (500 nM) or zymosan (500 μg/mL) on microglial O_2^- production measured as the reduction of NBT. Data are the mean values ±SE of four independent measurements in a representative of three experiments. ***p<0.001 vs. Hypo. **(b)** PMA (500 nM) had no effect on hypoosmotic D-[^{3}H]aspartate release from primary microglia. Data are the mean values ±SE of six experiments
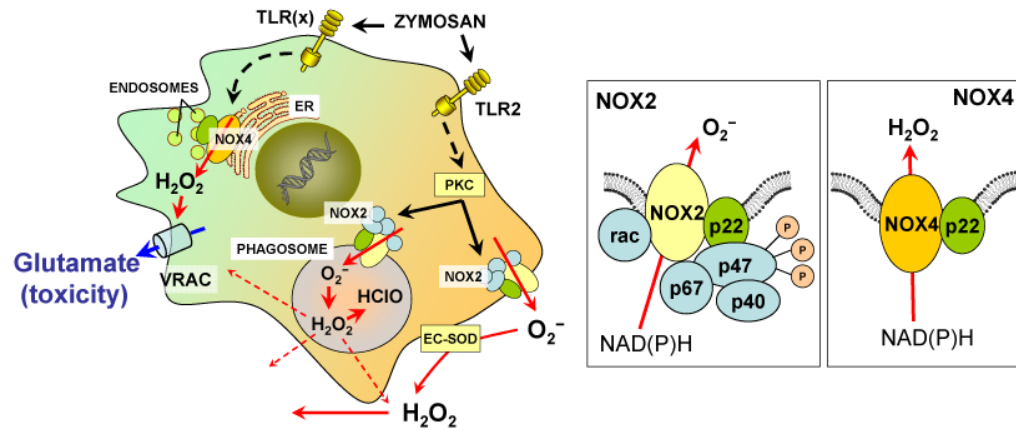
Fig. 10. Schematic representation of the hypothetical mechanisms contributing to the effects of zymosan on production of ROS and VRAC activity in rat microglial cells

Zymosan activates TLR2 and perhaps another unidentified subclass of the TLR receptors. TLR2 activation stimulates cytosolic PKC via several additional steps (not shown). PKC phosphorylates regulatory cytosolic subunit p47^{phox} (see NOX2 box on the right). Phosphorylation of p47^{phox} prompts assembly of the enzymatically active NOX2 complex, which includes membrane proteins NOX2 (gp91^{phox}) and p22^{phox}, as well as cytosolic p47^{phox}, p67^{phox}, p40^{phox}, and the small GTPase rac. An active NOX2 complex resides in plasmalemmal and phagocytic membranes and produces $O_2{}^-$, which is released to the extracellular space or into phagosome. An alternative signaling pathway causes activation of NOX4 via unidentified enzymatic steps. NOX4 is likely localized in endoplasmic reticulum and/or endosomes and does not require cytosolic regulatory subunits for its activity (see NOX4 box on the right). NOX4 produces predominantly H_2O_2, rather than $O_2{}^-$. The difference in the location of the ROS production and/or the nature of generated ROS likely determines differential contribution of NOX4 and NOX2 to the activation of VRAC by zymosan. Abbreviations: ER, endoplasmic reticulum; ES-SOD, extracellular superoxide dismutase; NOX, NAD(P)H oxidase, PKC, protein kinase C, TLR, Toll-like receptors. See text for additional details.

Table 1

Species specific primers for RT-PCR analysis of NOX isoform expression in microglia

NOX isoform	PCR primers		Predicted product (bp)
	Sense	Antisense	
NOX1	AAGCCATTGGATCACAACCT	GGCTTCTACTGTAGCGTTCA	115
NOX2	CATAAGATGGTAGCTTGGAT	GAGTGCTACTGAATATGGGT	123
NOX3	AAGCCATTGGATCACAACCT	GGCTTCTACTGTAGCGTTCA	108
NOX4	GCCTAGGATTGTGTTTGAGC	CGAAGGTAAGCCAGGACTGT	100