Research Paper

Reversed Na+/Ca2+ Exchange Contributes to Ca2+ Influx and Respiratory Burst in Microglia

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ABSTRACT
Phagocytosis and the ensuing NADPH-mediated respiratory burst are important aspects of microglial activation that require calcium ion (Ca2+) influx. However, the specific Ca2+ entry pathway(s) that regulates this mechanism remains unclear, with the best candidates being surface membrane Ca2+-permeable ion channels or Na+/Ca2+ exchangers. In order to address this issue, we used quantitative real-time RT-PCR to assess mRNA expression of the Na+/Ca2+ exchangers, Slc8a1-3/NCX1-3, before and after phagocytosis by rat microglia. All three Na+/Ca2+ exchangers were expressed, with mRNA levels of NCX1 > NCX3 > NCX2, and were unaltered during the one hour phagocytosis period. We then carried out a biophysical characterization of Na+/Ca2+ exchanger activity in these cells. To investigate conditions under which Na+/Ca2+ exchange was functional, we used a combination of perforated patch-clamp analysis, fluorescence imaging of a Ca2+ indicator (Fura-2) and a Na+ indicator (SBFI), and manipulations of membrane potential and intracellular and extracellular ions. Then, we used a pharmacological toolbox to compare the contribution of Na+/Ca2+ exchange with candidate Ca2+-permeable channels, to the NADPH-mediated respiratory burst that was triggered by phagocytosis. We find that inhibiting the reversed mode of the Na+/Ca2+ exchanger with KB-R7943, dose dependently reduced the phagocytosis-stimulated respiratory burst; whereas, blockers of store-operated Ca2+ channels or L-type voltage-gated Ca2+ channels had no effect. These results provide evidence that Na+/Ca2+ exchangers are potential therapeutic targets for reducing the bystander damage that often results from microglia activation in the damaged CNS.

ABBREVIATIONS
CR3, complement receptor 3; DPI, diphenylene iodonium; FBS, fetal bovine serum; HPRT1, hypoxanthine guanine phosphoribosyl transferase; IFNgα, interferon-gamma; IL1β, interleukin 1 beta; NADPH, nicotinamide adenine dinucleotide phosphate; NCX, Na+/Ca2+ exchanger; NMDG+, n-methyl d-glucamine; qRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction; SERCA, smooth endoplasmic reticulum calcium ATPase; SOC, store-operated Ca2+ channels; TNFα, tumor necrosis factor alpha

INTRODUCTION
As the resident immune cells of the CNS, microglia respond to damage or disease by undergoing a complex process of activation, in which dramatic changes in gene expression are accompanied by up-regulated cellular functions, including proliferation, phagocytosis, and production or release of reactive oxygen and nitrogen species, interleukins, cytokines and chemokines (recent reviews in refs. 1–12). Despite the presence in microglia of numerous receptor/ligand interactions that can elevate intracellular Ca2+ (Ca2+i), and the importance of Ca2+i in many aspects of microglial activation (reviewed in refs. 13–17), there is a dearth of information about which influx pathway(s) regulate which microglial functions. While it is often assumed that such responses are mediated by store-operated Ca2+ permeable channels, the presence of functional Na+/Ca2+ exchangers and members of the Slc8a/NCX family in microglia (reviewed in ref. 18) broadens the possibilities and deserves further consideration. In the normal mode, Na+/Ca2+ exchangers cause Ca2+ efflux, but under certain conditions they can reverse to mediate Ca2+ entry. The objective of this study of rat microglia was to assess the conditions under which...
reversed Na\(^+/\)Ca\(^{2+}\) exchange occurs and its potential contribution to the NADPH-dependent respiratory burst that accompanies phagocytosis. Reactive oxygen species liberated by the respiratory burst help degrade engulfed cellular debris but their extracellular release can also damage bystander cells, including neurons\(^{19-22}\) (reviewed in ref. 23), especially when combined with nitric oxide to produce the membrane-damaging peroxynitrite.

We used real-time quantitative RT-PCR to determine which Slc8a/NCX genes are expressed and to compare their mRNA expression levels. Having observed that un-stimulated microglia can display depolarization-enhanced Ca\(^{2+}\) entry with several of the salient features of reversed-mode Na\(^+/\)Ca\(^{2+}\) exchange, we then characterized conditions under which such Ca\(^{2+}\) entry occurs and directly assessed its voltage dependence. While it was not technically feasible to analyze Ca\(^{2+}\) influx during the respiratory burst that accompanies phagocytosis, we used KB-R7943 to block activity of the reversed-mode Na\(^+/\)Ca\(^{2+}\) exchanger, in order to assess its role in this important aspect of microglial activation. Our results provide support for testing inhibitors of Na\(^+/\)Ca\(^{2+}\) exchangers in CNS pathologies in which damage by oxygen free radicals has been implicated.

### MATERIALS AND METHODS

**Microglia cultures.** Microglia were isolated from brains of 2–3 day-old Wistar rats as we have previously described.\(^{24-26}\) In brief, rat pups were sacrificed by cervical dislocation in accordance with guidelines from the Canadian Institutes of Health Research and the University Health Network. After carefully removing the meninges, whole brain tissue was mashed through a stainless steel sieve (100 mesh; Tissue Grinder Kit #CD-1; Sigma-Aldrich; Oakville, ON, Canada), and then pelleted, resuspended and seeded into flasks with Minimal Essential Medium (MEM) containing 5% fetal bovine serum (FBS), 5% horse serum, and 100 μM gentamycin (all from Invitrogen, Burlington, Canada). Two days later, cellular debris, non-adherent cells, and supernatant were removed and fresh medium was added to the flask. The mixed cultures were allowed to grow for 7–10 days and then shaken for 4 h on an orbital shaker at 8-10 Hz in a standard tissue culture incubator. The supernatant containing detached microglia was centrifuged and the cell pellet was resuspended for counting, and then plated at 3.5 x 10⁴ cells per 15 mm diameter glass coverslip for electrophysiology. Before experiments, the plated microglia were cultured for 1–3 days in MEM with 100 μM gentamycin, and a reduced serum concentration (2% FBS), 1 CaCl\(_2\), 5 glucose, and 10 HEPES, adjusted to pH 7.4 (with NaOH) to ~300 mOsm with sucrose. For ion-substitution experiments, NaCl was partly or completely replaced with KCl, NMDG-Cl or LiCl, as indicated. For the nominally Ca\(^{2+}\)-free solutions, CaCl\(_2\) was omitted. EGTA was not added because we previously found that chelating all extracellular Ca\(^{2+}\) can promote spontaneous Ca\(^{2+}\) depletion,\(^{31}\) which would compromise calibrating microglial Ca\(^{2+}\) levels from dye measurements. Images were acquired at room temperature using a Nikon Diaphot inverted microscope, Retiga-EX camera (Q-Imaging, Burnaby, BC, Canada), DG-4 arc lamp and excitation wavelength changer (Sutter Instruments, Novato, CA), and Northern Eclipse image acquisition software (Empix Imaging, Mississauga, ON, Canada).

**Single-cell fluorescence imaging.** Microglia on coverslips were mounted in a perfusion chamber (Model RC-25, Warner Instruments, Hamden, CT) and the tissue culture medium was replaced with standard bath solution containing (in mM): 135 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 5 glucose, and 10 HEPES, adjusted to pH 7.4 (with NaOH) and to ~300 mOsm with sucrose. For ion-substitution experiments, NaCl was partly or completely replaced with KCl, NMDG-Cl or LiCl, as indicated. For the nominally Ca\(^{2+}\)-free solutions, CaCl\(_2\) was omitted. EGTA was not added because we previously found that chelating all extracellular Ca\(^{2+}\) can promote spontaneous Ca\(^{2+}\) depletion,\(^{31}\) which would compromise calibrating microglial Ca\(^{2+}\) levels from dye measurements. Images were acquired at room temperature using a Nikon Diaphot inverted microscope, Retiga-EX camera (Q-Imaging, Burnaby, BC, Canada), DG-4 arc lamp and excitation wavelength changer (Sutter Instruments, Novato, CA), and Northern Eclipse image acquisition software (Empix Imaging, Mississauga, ON, Canada).

To measure intracellular Ca\(^{2+}\), microglia were loaded (~30 min, room temperature) with 3.5 μg/ml Fura-2-AM (Invitrogen, Burlington, ON, Canada) made in the indicated bath solution. Images were acquired at 340 and 380 nm excitation wavelengths, and ratios were obtained using a 505 nm dichroic mirror and 510 nm emission filter. In the representative figures, Fura-2 data are presented as the 340/380 nm ratio, and the summarized data in the text are presented as intracellular Ca\(^{2+}\) levels, calibrated (as previously

### Table 1 Sequences of primers used for quantitative RT-PCR analysis

| Primer | Accession # | Sequence |
|--------|-------------|----------|
| HPRT1 (housekeeping) | NM_012583.2 | F: CAGTACAGCCCCCAATATTGGT  
R: CAAGGCGCATACCCACCAACA |
| NCX1/Slcb8a1 | NM_019268 | F: GATGAATGTGGGGAGGAA  
R: TCTGTAGTGGGGAGCAAGG |
| NCX2/Slcb8a2 | NM_078619 | F: GTCAAGGCTTTCGGGAGCAT  
R: AAAGAACACGGCTGGAGCATG |
| NCX3/Slcb8a3 | NM_078620 | F: CAAAGAACCGCGGACGAT  
R: TCTCCCGTTCCTCTACCA |
| CR3 | NM_012711 | F: TGCAGGACTGCGGGACAC  
R: CTCCCGAGACCTGTGTT |
| K\(_v\)1.3 | M30312 | F: GCTTCGCCGACTTTCAAG  
R: TGGTCTGCCACTGAAAGT |

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incubation at 37°C, 100 nM free Ca2+, 10 HEPES, 2 MgATP, adjusted to pH 7.2 with KOH, (10 mg/ml) in 50% rat serum (37°C, 1 h), washing (3x in PBS) and that contained 200 M amphotericin (Sigma-Aldrich) and (in mM): 100 K aspartate, 40 KCl, 1 MgCl2, 1 CaCl2, 10 EGTA (~20 nM free Ca2+), 10 HEPES, 2 MgATP, adjusted to pH 7.2 with KOH, osmolarity 280–300 mM. To test the effect of high intracellular Na+ in perforated patch recordings, the pipette solution contained 135 mM Na aspartate instead of K aspartate. Pipettes with resistances of 4–6 MΩ were pulled from borosilicate glass (WPI, Sarasota, FL). Patch clamp recordings were made at room temperature with a Multiclamp 700A patch-clamp amplifier (Molecular Devices, Sunnyvale, CA), filtered at 5 kHz, and compensated on-line for capacitance and series resistance. Data were digitized and acquired using a Digidata 1322 board with pCLAMP ver9.0 (Molecular Devices) and were analyzed using Origin ver7.0 (OriginLabs, Northampton, MA). Liquid-liquid junction potentials were calculated using the utility in pCLAMP, confirmed by measuring the values using a 3M KCl electrode,33 and subtracted before data analysis. After obtaining a giga-ohm seal, amphotericin caused a gradual decrease in series resistance, and when it reached <100 MΩ, experiments were begun. Fura-2 images were acquired every 2–3 s, and the excitation shutter was closed between acquisitions to prevent bleaching.

**RESULTS**

Evidence for spontaneous reversal of Na+/Ca2+ exchange in rat microglia. As a first step in analyzing the pathways of Ca2+ entry, we examined the effect of membrane potential on the rise in internal Ca2+ (Ca2+), caused by depletion of Ca2+ stores (Fig. 1). When Fura-2AM-loaded microglia were briefly exposed to the SERCA pump inhibitor (1 μM thapsigargin) in the presence of 1 mM external Ca2+, a Ca2+ rise was observed in all of the thousands of cells examined. In this example, all microglia (n = 26) showed an initial Ca2+ rise that is characteristic of release from internal stores and subsequent activation of store-operated Ca2+ (SOC) channels. We previously showed, by non-invasive imaging of membrane potential, that 55 mM external K+ (K+,) depolarizes the cells from about -50 mV to about -15 mV.26 When subsequently depolarized by elevating [K+]o to 55 mM, most microglia (21/26 cells) responded with a decrease in Ca2+, which rebounded when normal [K+]o was restored, and then rapidly decreased when external Ca2+ was removed. These responses are diagnostic of store-operated Ca2+ entry (reviewed in refs. 35–37), which requires external Ca2+ and decreases when the driving force is reduced by depolarization. However, in -10% of the optical fields, some microglia responded with a rise in Ca2+, in response to depolarization. In this experiment, for example, 5/26 microglia showed a further transient Ca2+ rise during the first application of the depolarizing 55 mM [K+]o solution. Since this only occurred in the first response to high [K+]o and the incidence was low and unpredictable, it was not feasible to assess the pharmacological profile of the underlying pathway. However, the simplest interpretation is that a second Ca2+ entry process was superimposed on normal store-operated Ca2+ entry.

The fact that this response occurred spontaneously, even though in a small proportion of cells, prompted us to study the second Ca2+ entry pathway in more detail. To isolate this pathway, we then omitted thapsigargin to avoid activating SOC, and used conditions that favor reversed Na+/Ca2+ exchange. For the experiment in Figure 1B, when a strongly depolarizing bath solution was added (140 [K+]o, Na+ free), Ca2+ rapidly rose in 3/8 microglia in the optical field, showing that store depletion is not essential. All 8 cells were microglia, as confirmed by labeling with tomato lectin, as in Figure 2A. Because reversed Na+/Ca2+ exchange can be evoked by decreasing external Na+, we asked whether this procedure could produce a Ca2+ rise. First, we showed that 2/20 microglia in one optical field produced a depolarization-induced Ca2+ rise in 55 mM [K+]o solution (Fig. 1C; only 4 cells shown). In the same two microglia, Ca2+ rise when external Na+ was reduced using a non-depolarizing solution; i.e., 50 mM Na+ replaced by n-methyl-d-glucamine (NMDG+). This experiment demonstrates a Ca2+ rise evoked by reducing external Na+, and an increased response by depolarizing the cells with high [K+]o.

**Relative transcript expression of Na+/Ca2+ exchangers in rat microglia.** Data in Figure 2 show quantitative real-time RT-PCR (qRT-PCR) results comparing expression levels of the Na+/Ca2+ exchangers, along with a membrane receptor involved in phagocytosis (CR3) and a K+ channel (K1.3) that is important for the microglial respiratory burst.24 Routine testing, like that in Figure 2A, showed that the cell cultures used in this study were 99-100% microglia. Based on their morphology,8 the untreated microglia were apparently in an ‘alert’ state, with most cells having long processes and amoeboid uropods. Phagocytic microglia are rod-shaped or nearly spherical.28 The relative order of mRNA expression for the Na+/Ca2+ exchangers was: NCX1 > NCX3 > NCX2, where ‘>’ denotes a significant difference from the preceding gene (p < 0.05). No significant changes were detected following a 1 h treatment with opsonized zymosan to stimulate phagocytosis and the respiratory
burst (see Fig. 7 for further explanation). Overall, there was a nearly 100-fold difference between NCX1 and NCX2 expression, and the NCX1 level was ~10% as high as complement receptor 3 (CR3) and the housekeeping gene (HPRT -1). Kᵥ1.3 expression was also relatively high, which is interesting because it not only contributes to functions of activated microglia,²⁴,³⁹ but it likely provides charge compensation for the depolarizing influence of Ca²⁺ entry, and thus maintains the Ca²⁺ driving force.²⁴ A useful calculation that relates the mRNA level to functional protein can be made for Kᵥ1.3; i.e., the number of active Kᵥ1.3 channels is about 500–1000/cell, estimated by dividing the whole-cell Kᵥ1.3 conductance (5–10 nS)²⁶ by the single channel Kᵥ1.3 conductance (~10 pS).⁴⁰

Figure 1. (Left) Evidence for spontaneous reversal of Na⁺/Ca²⁺ exchange in rat microglia. In this, and all subsequent figures, the Fura-2 ratio indicates changes in intracellular Ca²⁺ ([Ca²⁺]i), a horizontal line shows the period when a pharmacological agent is present, and altered bath solutions are shown as pale-coloured boxes. Unless otherwise indicated, the bath contained standard solution (see Methods). (A) Two types of Ca²⁺ rise can occur and show opposite responses to depolarization. In this experiment, Fura-2 loaded microglia were treated with the SERCA pump inhibitor, thapsigargin (1 μM, ~100 s) to deplete intracellular Ca²⁺ stores, while cells were exposed to standard bath solution containing (in mM): 1 Ca²⁺, 135 Na⁺, 5 K⁺ (see Methods). During the plateau phase, a moderately depolarizing high external K⁺ ([K+]o) solution was perfused in; i.e., 50 mM Na⁺ replaced by K⁺ (total [K+]o 55 mM). The first time [K+]o was elevated, Ca²⁺ decreased in 21/26 microglia (solid trace is average response), but rose in 5/26 cells (dotted trace). Subsequent applications of either Ca²⁺ free or high [K+]o solutions evoked a Ca²⁺ decrease in all microglia. (B) A depolarization-induced Ca²⁺ rise can be seen without depleting the Ca²⁺ stores; i.e., without thapsigargin. When standard bath solution was substituted with a strongly depolarizing solution ([K+]o 140 mM, no Na⁺) Ca²⁺ increased in 3/8 microglial cells in the field. (C) The Ca²⁺ rise is enhanced by, but does not require depolarization. When standard bath solution was replaced with the moderately depolarizing 55 mM [K+]o solution, 2 cells responded with a large Ca²⁺ rise, which decreased rapidly when the standard bath solution was restored. The other 18 cells in the field did not respond (for clarity, only 2 non-responding cells are shown). A smaller Ca²⁺ rise in the same 2 cells occurred when a non-depolarizing bath solution with reduced Na⁺ was perfused in (50 mM Na⁺ replaced with NMDG⁺).

Figure 2. Relative transcript expression of Na⁺/Ca²⁺ exchangers (Slc8a1-3) in rat microglia. (A) A typical culture of rat microglial cells used for quantitative real-time RT-PCR (qRT-PCR). Cells were labeled with FITC-conjugated tomato lectin (scale bar, 20 μm). (B) Relative mRNA expression was monitored by qRT-PCR, and standardized to the housekeeping gene, HPRT-1 (set to 1.0). Comparisons were made between unstimulated microglia (closed bars) and microglia after exposure for 1 h to opsonized zymosan (hatched bars). Values shown are mean ± SEM from 4 mRNA preparations made from separate batches of microglia isolated from different rat litters. Statistical differences were assessed with one way ANOVA, followed by Tukey’s test for multiple comparisons, and are indicated as *p < 0.05, **p < 0.01, ***p < 0.001.
Mean resting value determined by calibrating the Fura-2 signal, and taking the bath concentrations of 135 mM Na\(^+\) and 1 mM Ca\(^{2+}\), the calculated \(E_{NCX}\) is +9 mV. But, if intracellular Na\(^+\) rises to as little as 11 mM, \(E_{NCX}\) becomes -52 mV in the standard bath. Furthermore, if external Na\(^+\) is reduced to 85 mM (as is frequently used experimentally; see Fig. 1), then \(E_{NCX}\) will become -26 mV if Na\(^+\) is 5 mM, and -87 mV if Na\(^+\) is 11 mM. Using a voltage-sensitive dye, we measured the resting potential to be about -50 mV; hence, it should be easy to reverse the driving force and evoke Ca\(^{2+}\) entry if the membrane potential becomes depolarized, or the Na\(^+\) gradient is reduced by increasing Na\(^+\), or experimentally reducing external Na\(^+\). It is thus not surprising that Ca\(^{2+}\) entry occurred in some microglia (Fig. 1) when they were moderately depolarized (55 mM K\(^+\)) or external Na\(^+\) was reduced to 85 mM.

Experiments shown in Figures 3, 4 and 6 exploit the ionophore, gramicidin, and in order to interpret the results it is first necessary to consider its properties. Gramicidin forms channels that are permeable to K\(^+\) and Na\(^+\); thus, it rapidly depolarizes cells and more slowly dissipates the transmembrane Na\(^+\) and K\(^+\) gradients. The rise in internal Na\(^+\) should reverse Na\(^+\)/Ca\(^{2+}\) exchangers and promote Ca\(^{2+}\) entry. In order to observe the onset of these effects, gramicidin was added after the Ca\(^{2+}\) imaging recordings were begun.

Properties of reversed Na\(^+\)/Ca\(^{2+}\) exchange revealed using the ionophore, gramicidin. The unexpected Ca\(^{2+}\) rises observed in unstimulated microglia that were consistent with reversed Na\(^+\)/Ca\(^{2+}\) exchange (Fig. 1), prompted us to design experimental conditions to reliably activate the transporter and facilitate more detailed studies of its properties. We exploited the expectation that the direction and magnitude of Na\(^+\)/Ca\(^{2+}\) exchange will depend on both ion gradients, and on the membrane potential, as follows. Under normal conditions, Na\(^+\)/Ca\(^{2+}\) exchangers mediate Na\(^+\) influx and Ca\(^{2+}\) efflux. They are electrogenic, exchanging 3 Na\(^+\) ions for each Ca\(^{2+}\) ion, and their reversal potentials can be calculated from: \(E_{NCX} = 3E_{Na} - 2E_{Ca}\), where \(E_{Na}\) and \(E_{Ca}\) are the Nernst potentials for Na\(^+\) and Ca\(^{2+}\). First, we calculated the predicted reversal potential under physiologically relevant conditions. Reported values for intracellular Na\(^+\) (Na\(^i\)) in microglia range from 2–7 mM under resting conditions, to 5–11 mM after stimulation (e.g., with glycine). To calculate \(E_{NCX}\) we first converted concentrations (c) to activities (\(a_{ion}\)) using the formula: \(a_{ion} = f_{ion}c_{ion}\), where \(\log f_{ion} = (-0.51z^2V_i) / (1+V_i)\), and \(I\) is ionic strength. Setting Na\(^i\), at 5 mM, and Ca\(^{2+}\), at 70 mM (the
Na+. Lower panel: When first added in a Na+‑free bath solution, gramicidin responses occurred (similar to the first responses in the upper panel). Changes in Ca2+ concentration were similar in all cells examined. This time course was entirely consistent with Li+ substitution. As expected, the Ca2+ rise in our experiment was smaller if Na+ was reduced to 85 mM rather than 0 mM. Together, all results in Figure 3 are consistent with reversal of a Na+/Ca2+ exchanger.

A rise in internal Na+ evokes reversed-mode Na+/Ca2+ exchange. Next, we directly examined the rate of rise in cytoplasmic Na+ in response to gramicidin and demonstrate that Ca2+ influx can be evoked in microglia by reducing the Na+ gradient. Intracellular Na+ was imaged with SBF1, and Fluo‑4 was used to simultaneously image Ca2+, because its excitation and emission wavelengths allow dual imaging. Figure 4A demonstrates that both intracellular ions rise and, as can be seen from the inflection points, the increase in intracellular Na+ (beginning after ~85 sec in this example) preceded the Ca2+ rise. NB: The slow drift in Ca2+ signal occurs because Fluo-4 does not collapse immediately, despite inhibiting the Na+/K+ ATPase with ouabain. Alternatively, some studies preincubate with gramicidin for several minutes to allow the cells to load with Na+ before recording rapid responses to changes in extracellular ions. The slow dissipation of the Na+ gradient is also consistent with the实验 in Figure 3D. When external Na+ was completely replaced with Li+, there was an immediate, large rise in Ca2+, due to the removal of Na+. This was followed within seconds by a relaxation of the Ca2+ signal in all cells examined. This time course was entirely consistent with Li+ influx and Na+ efflux through gramicidin channels, and the failure of Li+ to support the reversed mode of the Na+/Ca2+ exchanger.

We did not examine the time course of the relaxation using longer experiments with Li+, but an earlier study applied gramicidin for > 10 min before testing Li+ substitution. As expected, the Ca2+ rise in our experiment was smaller if Na+ was reduced to 85 mM rather than 0 mM. Together, all results in Figure 3 are consistent with reversal of a Na+/Ca2+ exchanger.

Figure 4. Elevated internal Na+ evokes a Ca2+ rise through reversed Na+/Ca2+ exchange. (A and B) Changes in intracellular Na+ correlate with Ca2+ entry. Simultaneous imaging of intracellular Ca2+ and Na+ in microglia loaded with Fluo‑4‑AM (490 nm excitation) and SBFI‑AM (ratio imaged at 340/380 nm excitation). In standard bath solution, 1 μM gramicidin elicited a rise in Na+ and then Ca2+. (A) Is on an expanded time scale to show the inflection point in the Na+ rise (dashed line). Removing external Na+ (B) evoked a further increase in Ca2+. (C) The Ca2+ rise requires intracellular Na+. Fura‑2 ratio imaging of intracellular Ca2+. Upper panel: Gramicidin (1 μM) evoked a Ca2+ rise that was greatly enhanced by removing external Na+. Lower panel: When first added in a Na+‑free bath solution, gramicidin did not evoke a Ca2+ rise. After external Na+ was restored, the normal responses occurred (similar to the first responses in the upper panel).

in Ca2+1, as expected for reversed Na+/Ca2+ exchange resulting from depolarization combined with a rise in intracellular Na+. Importantly, experiments in Figure 3B–D show that the gramicidin-induced Ca2+ rise does not require depletion of intracellular Ca2+ stores and activation of store-operated channels. That is, adding gramicidin alone caused a substantial Ca2+ rise (Fig. 3B), and although the individual cell responses varied in amplitude (Fig. 3C) they were qualitatively similar in all cells examined. Changes in Ca2+ concentration were calculated (from Fura-2 signals) in several such experiments: in standard bath solution, Ca2+ increased from 68 ± 5 nM to 285 ± 18 nM at 100 s after gramicidin application (n = 115 cells). This Ca2+ rise required influx, but not release from internal stores, since it was fully prevented by removing extracellular Ca2+ (inset in Fig. 3C). Importantly, exposure to gramicidin rendered all microglia responsive, such that a subsequent exposure to a depolarizing solution with reduced external Na+ (Fig. 3B and C) evoked a Ca2+ rise in all cells examined (> 200 cells).

There was a delay after adding gramicidin until a rise in Ca2+, was seen (~30 sec to > 60 sec), which reflects several processes: exchanging the bath solution (~10 sec), incorporation and accumulation of gramicidin in cell membranes, and diffusion-mediated run-down of the ion gradients. The latter two processes will be affected by activity of pumps and other transporters and by cell surface-to-volume ratio, which is highly variable for microglia that range from highly ramified ‘resting’ cells, to ‘amoeboid’ cells that can bear long processes, to rounded up ‘fully activated’ cells. Others have observed a relatively slow equilibration of internal Na+ and a delayed rise in Ca2+, after adding gramicidin, as much as 3–4 min. A more direct study using gramicidin to calibrate the Na+‑sensitive dye, SBFI, showed a variable time course. When external Na+ was reduced the gradient did not collapse immediately, despite inhibiting the Na+/K+ ATPase with ouabain. Alternatively, some studies preincubate with gramicidin for several minutes to allow the cells to load with Na+ before recording rapid responses to changes in extracellular ions. The slow dissipation of the Na+ gradient is also consistent with the experiment in Figure 3D. When external Na+ was completely replaced with Li+, there was an immediate, large rise in Ca2+, due to the removal of Na+. This was followed within seconds by a relaxation of the Ca2+ signal in all cells examined. This time course was entirely consistent with Li+ influx and Na+ efflux through gramicidin channels, and the failure of Li+ to support the reversed mode of the Na+/Ca2+ exchanger. We did not examine the time course of the relaxation using longer experiments with Li+, but an earlier study applied gramicidin for > 10 min before testing Li+ substitution. As expected, the Ca2+ rise in our experiment was smaller if Na+ was reduced to 85 mM rather than 0 mM. Together, all results in Figure 3 are consistent with reversal of a Na+/Ca2+ exchanger.

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Reversal of the Na+/Ca2+ exchanger, and was not a direct result of removing external Na+. When gramicidin was added in the absence of external Na+ (lower panel), removing external Na+ did not evoke a Ca2+ rise. This is most likely because internal Na+ was depleted by efflux through the Na+ permeable gramicidin channels (as in Fig. 4B). However, after allowing time for internal Na+ to be replenished in standard bath solution, normal Ca2+ responses were restored. Note that in control experiments with normal bath Na+ (upper panel); gramicidin produced the usual responses to removal of external Na+ in cells from the same batches.

The voltage-dependence of Ca2+ entry mediated by Na+/Ca2+ exchange. To directly determine the effect of membrane potential (V_m) on Ca2+ entry through reversed-mode Na+/Ca2+ exchange, the Fura-2 signal was imaged while the membrane potential was controlled in current clamp recordings. The perforated patch configuration was used with amphotericin, which allows whole-cell recording by forming channels that are permeable to monovalent ions (Na+, K+, Cl-) but impermeable to divalent cations. Thus, in order to promote reversal of the Na+/Ca2+ exchanger, intracellular Na+ can be elevated in each patch-clamped cell using a pipette solution containing 135 mM Na+. Changes in intracellular Ca2+ were evoked by voltage steps (to -80, -50, -10 and +30 mV; lower panel). First, the bath contained standard solution, then external Na+ was reduced to 85 mM, and finally all external Na+ was replaced by K+. Vertical dashed lines show selected voltage changes aligned with Ca2+ responses.

Figure 5. Voltage-dependence of Ca2+ entry through reversed-mode Na+/Ca2+ exchange. Amphotericin-mediated perforated patch recordings (explained in Results) were combined with simultaneous Ca2+ imaging of Fura-2 AM-loaded cells. To increase intracellular Na+ in the patch-clamped cell, the pipette solution contained 135 mM Na+. Changes in intracellular Ca2+ were evoked by voltage steps (to -80, -50, -10 and +30 mV; lower panel). First, the bath contained standard solution, then external Na+ was reduced to 85 mM, and finally all external Na+ was replaced by K+. Vertical dashed lines show selected voltage changes aligned with Ca2+ responses.

Reversed Na+/Ca2+ exchange contributes to the phagocytosis-mediated respiratory burst. The evidence presented thus far is that reversed Na+/Ca2+ exchange can be readily evoked in microglia under the appropriate conditions; i.e., depolarization, increased intracellular Na+ or reduced external Na+. Before addressing functional roles of the exchanger, it was necessary to identify a pharmacological inhibitor. KB-R7943 is a broad-spectrum NCX blocker that preferentially blocks the reversed mode of the exchangers, with a reported IC50 of ~5 μM. First, we elicited stereotypical Ca2+ rises in...
Role of Reversed Na+/Ca2+ Exchange in Microglia

Figure 7. Role of reversed Na+/Ca2+ exchange in the phagocytosis-mediated respiratory burst. (A) Role of Ca²⁺ entry through reversed Na+/Ca2⁺ exchange. An NADPH-mediated respiratory burst was evoked in response to phagocytosis of opsonized zymosan (100 µg/ml; 1 h, 37°C), and monitored as a fluorescence increase in microglia labeled with 10 µM dihydroethidium (see Methods). After subtracting the fluorescence signal from unstimulated cells, the signal was normalized to the value in drug-free controls. Values are mean ± SEM for the number of separate experiments indicated on each bar (*p < 0.05). When used, each of the following compounds was added to the bath at the same time as opsonized zymosan: the NADPH inhibitor, diphenylene iodonium (DPI; 200 nM); the Ca²⁺ chelator, EGTA (10 mM); and the Na⁺/Ca²⁺ exchanger inhibitor, KB-R7943 (20 µM). (B) Dose-dependence of inhibition by KB-R7943 (mean ± SEM, n = 3). The respiratory burst was monitored at different concentrations of KB-R7943. A curve was fit to: percent block = 1/[1+IC₅₀/[KBKBR]], yielding an IC₅₀ of 5.0 µM.

control recordings from several cell batches (Fig. 6A, upper panel), and then showed that 10 µM KB-R7943 prevented the response to removing external Na⁺ (lower panel) in aliquots from the same cell batches. It has been suggested that microglia express functional voltage-dependent L-type Ca²⁺. Results above (Fig. 5) appeared to rule out a contribution of Ca²⁺ channels to the Ca²⁺ rise when intracellular Na⁺ is elevated. In Figure 6B, a contribution of L-type Ca²⁺ channels is ruled out because the typical Ca²⁺ responses to gramicidin were unaffected by nifedipine (5 µM shown or 100 µM not shown).

To address the physiological importance of Na⁺/Ca²⁺ exchange-mediated Ca²⁺ influx, we asked whether it plays a role in the respiratory burst, which can render activated microglia neurotoxic. Phagocytosis was stimulated using opsonized zymosan, and the ensuing respiratory burst was monitored as superoxide production, and shown to be mediated by NADPH-oxidase, because it was inhibited by diphenylene iodonium (DPI). External Ca²⁺ was required, since superoxide production was inhibited by chelating Ca²⁺ in the bath with 10 mM EGTA. Most importantly, KB-R7943 dose-dependently reduced the respiratory burst, with an IC₅₀ of 5.0 µM (Fig. 7B); the same as the published value for inhibiting Na⁺/Ca²⁺ exchangers (see above). KB-R7943 has been reported to inhibit store-operated Ca²⁺ (SOC) channels; however, this side effect was ruled out because the SOC inhibitor, SKF-96365, had no effect (Fig. 7A). Finally, KB-R7943 has been reported to displace some L-type Ca²⁺ channel blockers (verapamil, diltiazem; but not the dihydropyridine, PN200-100) from binding sites in rat cerebral cortex. However, we ruled out an involvement of L-type Ca²⁺ channels in the Ca²⁺ rise (Fig. 6B), and found that the respiratory burst was not reduced by nifedipine. That is, superoxide production was 96.9 ± 8.0% of the control value with 5 µM and 94.9 ± 6.9% with 25 µM nifedipine (p > 0.2; n = 3 cultures). KB-R7943 does not inhibit several other ion transport molecules that are potentially involved in the respiratory burst in microglia; such as, Na⁺/H⁺ exchanger, Na⁺/K⁺ ATPase or Ca²⁺ ATPases.

**DISCUSSION**

This study provides direct evidence that Ca²⁺ entry by reversed Na⁺/Ca²⁺ exchange can occur under normal conditions and can contribute to microglial activation. Using single-cell Ca²⁺ imaging and perforated patch-clamp recording, we demonstrated the salient features of this Na⁺/Ca²⁺ exchange. The significant findings are: (i) The rise in intracellular Ca²⁺ (Ca²⁺i) was abolished by removing external Ca²⁺, which demonstrates that the source is Ca²⁺ influx, not internal release. In addition, this Ca²⁺ influx pathway did not require depletion of Ca²⁺ stores, and thus differs from store-operated Ca²⁺ channels. (ii) Ca²⁺ entry through reversed Na⁺/Ca²⁺ exchange was enhanced by depolarization, which potentially resolves discrepancies in the literature concerning the existence of depolarization-activated L-type Ca²⁺ channels in microglia. (iii) Conditions were delineated under which reversed Na⁺/Ca²⁺ exchange was induced in all of the thousands of microglia examined. This occurred when they were loaded with Na⁺ using the cationophore, gramicidin (confirmed with a Na⁺-sensitive dye), or when some extracellular Na⁺ was replaced with a non-transported cation, or when the microglia were moderately depolarized. (iv) Another key observation, which is consistent with reversed Na⁺/Ca²⁺ exchange, is the link between efflux of internal Na⁺ and influx of external Ca²⁺. That is, there was a time lag during which internal Na⁺ rose, and it preceded the Ca²⁺i rise, which was also prevented if intracellular Na⁺ was depleted. (v) The relative transcript levels of Na⁺/Ca²⁺ exchangers was NCX1 > NCX3 > NCX2, and was unaltered during the time required for the microglia to phagocytose opsonized zymosan. (vi) Finally, Ca²⁺ influx through reversed Na⁺/Ca²⁺ exchange contributed to a generally neurotoxic microglia function, the NADPH-mediated respiratory burst that accompanies phagocytosis. That is, Ca²⁺ influx through this pathway and phagocytosis were reduced by the same concentrations of the Na⁺/Ca²⁺ exchange inhibitor, KB-R7943.

Based on these properties, Ca²⁺ entry through reversed Na⁺/Ca²⁺ exchange will likely be most important under pathological conditions that favor microglia depolarization or an elevation in either internal Na⁺ or external K⁺. Interestingly, in a small proportion
of cultured microglial cells we found reversed \( \text{Na}^+/\text{Ca}^{2+} \) exchange without any manipulations. This observation may indicate that some microglia have higher initial \( \text{Na}^+ \) levels, depolarized membrane potentials or both. Of note, the likelihood of observing reversed \( \text{Na}^+/\text{Ca}^{2+} \) exchange activity and the resulting rise in intracellular \( \text{Ca}^{2+} \) were dramatically increased when internal \( \text{Na}^+ \) was elevated. Especially under pathological conditions, several pathways can potentially cause a rise in cytoplasmic \( \text{Na}^+ \). For instance, microglia have \( \text{Na}^+ \)-permeable ion channels, including non-selective cation channels, and ionotropic purinergic receptors (reviewed in refs. 15 and 53) that can be activated by ATP release from damaged cells. Microglia also robustly express functional TRPM7 channels, which can conduct large inward monovalent currents; e.g., when extracellular pHi is reduced to levels that have been observed during tissue injury, ischemia, repetitive nerve activity or seizures. In addition, activated microglia express high levels of \( \text{Na}^+ \)/glutamate co-transporters which, in astrocytes, cause large glutamate-induced elevations in internal \( \text{Na}^+ \). Extracellular glycine, which increases in ischemia, neurotrauma and epilepsy, elevates internal \( \text{Na}^+ \) in microglia through \( \text{Na}^+ \)-coupled neutral amino acid transporters. In astrocytes, ischemia and reperfusion dramatically increase internal \( \text{Na}^+ \) through a \( \text{Na}^+ / \text{K}^+ / \text{Cl}^- \) cotransporter, and a similar mechanism might exist in microglia. Thus, there are numerous conditions under which \( \text{Na}^+ \) influx should elevate internal \( \text{Na}^+ \) in microglia, causing reversal of \( \text{Na}^+ /\text{Ca}^{2+} \) exchange and consequent \( \text{Ca}^{2+} \) entry (see Results for sample calculations). Furthermore, reversal of \( \text{Na}^+ /\text{Ca}^{2+} \) exchange will be facilitated by some changes in extracellular ions that occur under a variety of pathological conditions. Changes in extracellular \( \text{K}^+ \) \( [\text{K}^+]_o \) are especially important because they can affect the membrane potential. After brain ischemia, \( [\text{K}^+]_o \) rises and \( [\text{Na}^+]_o \) falls to levels comparable to those used in the present study; e.g., after 15 min of ischemia \( [\text{K}^+]_o \) rises to 42 mM and \( [\text{Na}^+]_o \) falls to 64 mM. Reversed \( \text{Na}^+/\text{Ca}^{2+} \) exchange is relevant to a broad range of CNS pathologies. It appears to mediate ‘\( \text{Ca}^{2+} \)-paradox injury’, which is delayed cell death due to a persistent rise in internal \( \text{Ca}^{2+} \) after cells are exposed to \( \text{Ca}^{2+} \)-free solutions, then reperfused with normal \( \text{Ca}^{2+} \). This process is often considered to be an in vitro model of cerebral ischemia/reperfusion injury because a similar decrease, followed by an increase in external \( \text{Ca}^{2+} \) has been seen after stroke. There are two families of \( \text{Na}^+/\text{Ca}^{2+} \) exchanger genes with several members each; the strictly \( \text{Na}^+/\text{Ca}^{2+} \) dependent, \( \text{SLC} 8 \text{a}/\text{NCX} \) family (usually called \( \text{Slc} 8 \text{a} \) in rodents) and the \( \text{K}^+ \) dependent \( \text{SLC} 24 \text{a}/\text{NCKX} \) family. Both families are expressed in the brain but their cellular distribution has not been fully characterized, and the precise gene (or genes) involved in brain injury is not known. All three members of the \( \text{Slc} 8 \text{a}1-3/\text{NCX}1-3 \) family are found in microglia (ref. 48 and present study), and the most highly expressed, \( \text{Slc} 8 \text{a}1/\text{NCX}1 \), is an important \( \text{Ca}^{2+} \) transporter in most cells (reviewed in ref. 61). Although we did not detect a change in their expression after inducing phagocytosis for one hour; pathological conditions, other stimuli and those acting over longer periods might affect expression. For instance, both \( \text{NCX} \) expression and \( \text{Ca}^{2+} \) signaling were found to be transiently up-regulated after treatment with IFNγ, a cytokine implicated in CNS pathologies such as stroke. In future, it would be interesting to determine if TNFα and IL1β, which evoke chronic \( \text{Ca}^{2+} \) elevations in microglia (reviewed in ref. 16) also up-regulate \( \text{Slc} 8 \text{a}1/\text{NCX} \) expression or activity. We had hoped to use siRNA-mediated knockdown to identify the specific \( \text{NCX} \) gene that mediates the reversed \( \text{Na}^+/\text{Ca}^{2+} \) exchange. If successful, this approach could also be used to assess roles of identified \( \text{Slc} 8 \text{a}1/\text{NCX} \) genes in microglia-mediated neurotoxicity, as we have previously done for microglial \( \text{K}^+ \) channels. However, we have found that siRNA mediated knockdown is extremely difficult and inefficient in primary microglial cells, and most transfection or injection treatments were toxic (unpublished results).

Numerous studies implicate oxygen free radicals in microglia-mediated neuron damage after stroke (reviewed in ref. 63). How, specifically, might reversed \( \text{Na}^+/\text{Ca}^{2+} \) exchange contribute to the respiratory burst? Ionic requirements for the NADPH-mediated respiratory burst in phagocytes are complex but worth considering when comparing the present results with previous work. Many studies use a phorbol ester to evoke a respiratory burst; however, in bypassing the phagocytosis receptors, downstream signaling mechanisms will likely be compromised. An important methodological difference is that in the present study, the respiratory burst was a consequence of phagocytosis. Under these conditions, external \( \text{Ca}^{2+} \) was required for superoxide production, and we provide the first evidence that reversed \( \text{Na}^+/\text{Ca}^{2+} \) exchange contributes to this respiratory burst in microglia. During the respiratory burst, depolarization and a rise in internal \( \text{Na}^+ \) have been observed, both of which should promote reversal of \( \text{Na}^+/\text{Ca}^{2+} \) exchange and consequent \( \text{Ca}^{2+} \) entry. The voltage dependence of reversed \( \text{Na}^+/\text{Ca}^{2+} \) exchange shown in the present study might explain earlier observations that the phorbol ester-induced respiratory burst is potentiated by high external \( \text{K}^+ \) which should depolarize the cells.

A second consideration is that in order to produce a respiratory burst, NADPH is converted to NADP+ and \( \text{H}^+ \), electrons are released and they combine with \( \text{O}_2 \) to form extracellular \( \text{O}_2^- \) (superoxide). In principle, the electron efflux could depolarize the cell sufficiently to reverse \( \text{Na}^+/\text{Ca}^{2+} \) exchange without a rise in internal \( \text{Na}^+ \). Concurrent with NADPH activation, \( \text{H}^+ \) is released to acidify the phagosomes. If activated at the plasma membrane, \( \text{Na}^+/\text{H}^+ \) exchangers will promote \( \text{Na}^+ \) influx while extruding \( \text{H}^+ \). One potential mechanism to compensate for depolarization and maintain the large driving force for electron efflux that drives NADPH oxidase, is to promote \( \text{K}^+ \) efflux through ion channels. Such a mechanism is consistent with the dramatically reduced respiratory burst when voltage-dependent (\( \text{K}^+ \)) or \( \text{Ca}^{2+} \)-dependent (\( \text{SK} \)) channels are blocked in microglia. In addition, \( \text{Cl}^- \) influx through anion channel could help counteract the depolarization resulting from electron efflux. We recently showed that anion channels are major determinants of the membrane potential in microglia and thus, should affect \( \text{Ca}^{2+} \) entry, and also contribute to phagocytosis. We recognize that quantitative and qualitative differences in the predominant ionic pathways might exist between different cell types and might also depend on the stimulus used. When the much larger respiratory burst is activated by phorbol esters in eosinophils or neutrophils, efflux through an \( \text{H}^+ \) selective channel appears to counteract the depolarization and remove excess internal \( \text{H}^+ \).

Based on the role of reversed \( \text{Na}^+/\text{Ca}^{2+} \) exchange in elevating \( \text{Ca}^{2+} \) and activating astrocytes, a potent inhibitor, SEA0400, was tested and found to improve the outcome in a rat model of transient ischemic stroke. The present results showing a role for reversed \( \text{Na}^+/\text{Ca}^{2+} \) exchange in the phagocytosis-mediated respiratory burst in...
microglia, supports testing specific inhibitors in models of a variety of CNS pathologies that involve release of potentially toxic free radicals from microglia.

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