K⁺ Efflux Agonists Induce NLRP3 Inflammasome Activation Independently of Ca²⁺ Signaling

Michael A. Katsnelson, L. Graham Rucker, Hana M. Russo and George R. Dubyak

*J Immunol* 2015; 194:3937-3952; Prepublished online 11 March 2015;
doi: 10.4049/jimmunol.1402658

http://www.jimmunol.org/content/194/8/3937

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2015/03/10/jimmunol.1402658.DCSupplemental

**References**

This article cites 61 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/194/8/3937.full#ref-list-1
K+ Efflux Agonists Induce NLRP3 Inflammasome Activation Independently of Ca2+ Signaling

Michael A. Katsnelson,* L. Graham Rucker,† Hana M. Russo,* and George R. Dubyak‡

Perturbation of intracellular ion homeostasis is a major cellular stress signal for activation of NLRP3 inflammasome signaling that results in caspase-1–mediated production of IL-1β and pyroptosis. However, the relative contributions of decreased cytosolic K+ concentration versus increased cytosolic Ca2+ concentration ([Ca2+]) remain disputed and incompletely defined. We investigated roles for elevated cytosolic [Ca2+] in NLRP3 activation and downstream inflammasome signaling responses in primary murine dendritic cells and macrophages in response to two canonical NLRP3 agonists (ATP and nigericin) that facilitate primary K+ efflux by mechanistically distinct pathways or the lysosome-destabilizing agonist Leu-Leu-O-methyl ester. The study provides three major findings relevant to this unresolved area of NLRP3 regulation. First, increased cytosolic [Ca2+] was neither a necessary nor sufficient signal for the NLRP3 inflammasome cascade during activation by endogenous ATP-gated P2X7 receptor channels, the exogenous bacterial ionophore nigericin, or the lysosomotropic agent Leu-Leu-O-methyl ester. Second, agonists for three Ca2+-mobilizing G protein–coupled receptors (formyl peptide receptor, P2Y2 purinergic receptor, and calcium-sensing receptor) expressed in murine dendritic cells were ineffective as activators of rapidly induced NLRP3 signaling when directly compared with the K+ efflux agonists. Third, the intracellular Ca2+ buffer, BAPTA, and the channel blocker, 2-aminoethoxydiphenyl borate, widely used reagents for disruption of Ca2+-dependent signaling pathways, strongly suppressed nigericin-induced NLRP3 inflammasome signaling via mechanisms dissociated from their canonical or expected effects on Ca2+ homeostasis. The results indicate that the ability of K+ efflux agonists to activate NLRP3 inflammasome signaling can be dissociated from changes in cytosolic [Ca2+] as a necessary or sufficient signal. The Journal of Immunology, 2015, 194: 3937–3952.

Interleukin-1β is a primary proinflammatory cytokine that activates the acute phase response, induces fever, promotes proliferation of neutrophils in the bone marrow, and stimulates adherence of leukocytes to the walls of blood vessels (1). Production of biologically active IL-1β requires its proteolytic processing (maturation) by caspase-1. Caspase-1 activation per se involves autolytic processing regulated by assembly of inflammasome signaling complexes (2). One major type of inflammasomes consists of the cytosolic pattern recognition receptor NLRP3, the adaptor protein ASC, and procaspase-1 (2, 3). Upon activation, NLRP3 monomers oligomerize into a ring-like structure that recruits ASC monomers to induce formation of ASC filaments or specks (4, 5). In turn, ASC filaments/specks recruit multiple procaspase-1 monomers to facilitate the induced proximity required for autocatalytic proteolysis into the 10- and 20-kDa subunits that assemble into the highly active tetramers of Caspase-1. Active caspase-1 mediates both the proteolytic maturation of IL-1β and its release via noncanonical secretion (2, 3). Caspase-1 also induces pyroptosis, a regulated cell death pathway characterized by the permeabilization of the plasma membrane that facilitates collapse of ionic and osmotic homeostasis and eventual cell lysis (6).

The assembly of NLRP3 inflammasomes can be activated by various exogenous stimuli, including extracellular ATP (via opening of P2X7 nonselective cation channel receptors), small microbial ionophores such as nigericin or gramicidin, and large bacterial pore-forming protein toxins (7, 8). All of these NLRP3-activating stimuli directly perturb the permeability of the plasma membrane to K+ with consequent reduction in cytosolic K+ concentration ([K+]). Multiple studies have identified decreased cytosolic [K+] as a necessary signal for the induction of NLRP3 inflammasome assembly by those stimuli that directly target plasma membrane permeability (9). Importantly, Muñoz-Planillo et al. (10) reported that the ability of particulate stimuli, such as monosodium urate, silica, and aluminum hydroxide, or small molecule lysosomotropic molecules, such as Leu-Leu-O-methyl ester (LLME), to activate NLRP3 inflammasomes secondary to lysosomal destabilization also requires efflux of K+. However, it remains unclear whether decreased cytosolic [K+] is a sufficient ionic signal in addition to being a necessary signal. In this regard, other studies have implicated elevations in cytosolic Ca2+ concentration ([Ca2+]i) in the NLRP3 inflammasome activation response to several stimuli, including nigericin and ATP-gated P2X7 receptor channels. Several reports indicated that caspase-1 activation and IL-1β release are suppressed in macrophages loaded with BAPTA, a chelator of cytosolic Ca2+ (11,12). Murakami et al. (13) found that NLRP3 inflammasome signaling in response to

Received for publication October 17, 2014. Accepted for publication February 7, 2015.

This work was supported by American Heart Association Predoctoral Fellowship 13PRE16860052 (to M.A.K.) and by National Institutes of Health Grants RO1-GM36387 (to G.R.D.), T32-AI089474 (to H.M.R.), T32-GM007250 (to M.A.K. and H.M.R.), T32-HL105338 (to M.A.K.), and R25-HL03152 (to L.G.R.).

Address correspondence and reprint requests to Dr. George R. Dubyak, Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106. E-mail address: george.dubyak@case.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: 2-APB, 2-aminoethoxydiphenyl borate; BMDC, bone marrow–derived DC; BMDM, bone marrow–derived macrophage; BSS, balanced salt solution; [Ca2+]i, cytosolic Ca2+ concentration; CacS, calcium-sensing receptor; DC, dendritic cell; DSS, dextran sodium sulfate; ER, endoplasmic reticulum; fluo-4/AM, fluo-4-acetoxyethyl ester; FPR, formyl peptide receptor; GPCR, G protein–coupled receptor; IP3, inositol trisphosphate; [K+], K+ concentration; LDH, lactate dehydrogenase; LLME, Leu-Leu-O-methyl ester; PLC, phospholipase C; WT, wild-type.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/525/00
nigericin and ATP was markedly attenuated by inhibitors of phospholipase C (U73122), IP3-gated Ca2+ release channels (xestospongin C), or store-operated Ca2+ entry (2-aminoethoxydiphenyl borate [2-APB]). Finally, agonists for the calcium-sensing G protein–coupled receptor (GPCR; CaSR) and CsA complement GPCR, which stimulate phospholipase C–mediated increases in cytosolic [Ca2+]2, have been reported to induce NLRP3 inflammasome–dependent IL-1β release from murine or human monocytes/macrophages (14–16).

These observations suggest that increased cytosolic [Ca2+]2 may activate NLRP3 inflammasome signaling either independently of, or synergistically with, decreased cytosolic [K+]K. However, no previous studies have directly measured changes in both cytosolic [Ca2+]2 and [K+]K in myeloid leukocytes under the experimental conditions routinely used to interrogate key reactions of the NLRP3 inflammasome signaling cascade. Moreover, the pharmacologic tools for investigating the role of Ca2+ signaling in inflammasome activation can affect the homeostasis of other valent cations, for example, Zn2+, or the activity of nonsellective channels permeable to both divalent and monovalent cations (17, 18). In this study, we investigated the role of elevated cytosolic [Ca2+]2 in NLRP3 activation in murine macrophages and dendritic cells (DC) with minimal use of pharmacologic inhibitors and by directly assaying changes in cytosolic [Ca2+]2 in response to two canonical NLRP3 agonists (ATP or nigericin) that facilitate K+ efflux by mechanistically distinct reactions (see Fig. 1A) (19, 20). Other experiments evaluated whether changes in cytosolic [Ca2+]2 signaling modulate NLRP3 inflammasome activation by lysosomal destabilization. We assessed possible effects of increased cytosolic [Ca2+]2 on several discrete phases of the NLRP3 inflammasome signaling cascade: formation of ASC oligomers, processing/release of caspase-1, processing/release of IL-1β, and kinetics of caspase-1–mediated pyroptosis. Our results indicate that the ability of K+ efflux agonists to activate NLRP3 signaling can be dissociated from changes in cytosolic [Ca2+]2. Moreover, nigericin-stimulated increases in cytosolic [Ca2+]2 were temporally correlated with the onset and kinetics of pyroptosis and were absent in DC isolated from Casp11−/− or Nlrp3−/− mice.

Materials and Methods

Reagents

Key reagents and their sources are as follows: Escherichia coli LPS serotype O111:B4 (List Biological Laboratories), PamC3Sk4 (Invivo-Gen), murine rTNF-α (PeproTech), murine rM-CSF (PeproTech), nigericin (Sigma-Aldrich), ATP (Sigma-Aldrich), ionomycin (LC Laboratories), N-formyl-Met-Leu-Phe (Sigma-Aldrich), UTP (Sigma-Aldrich), H-Leu-Leu-OMe-HBr (Bachem), lidocaine (Sigma-Aldrich), R568 (Tocris Biosciences), taspargin (LC Laboratories), diisouminalyl sulbrate (DSS; Sigma-Aldrich), BAPTA-AM (Molecular Probes), 2-APB (Tocris Bioscience), anti-caspase-1 (g20) mouse mAb (Casper-1) (Adiogen), anti-ASC rabbit polyclonal Ab (Ab-N), and all HRP-conjugated secondary Abs (Santa Cruz Biotechnology), murine IL-1β ELISA kit (R&D Systems), fluo-4–acetoxymethyl ester (flu-4–AM; Life Technologies), Pluronic F-127 (Molecular Probes), probenecid (Sigma-Aldrich), propidium iodide (Life Technologies), lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche). Anti–IL-1β mouse mAb was provided by the Biological Resources Branch, National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD).

Murine models

Wild-type (WT) C57BL/6 mice were purchased from Taconic. Mice lacking both caspase-1 and caspase-11 on a C57BL/6 background (Casp11−/−−/−) have been previously described (21). Nlrp3−/− mice were provided by Dr. A. Hise (Case Western Reserve University). All experiments and procedures involving mice were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University. Bone marrow–derived DC (BMDC) and bone marrow–derived macrophages (BMDM) were isolated from 9- to 12-wk-old mice. Mice were euthanized by CO2 inhala-

Ca2+-INDEPENDENT NLRP3 INFLAMMASOME ACTIVATION

Prior to experimental treatments, BMDC-containing TC plates were centrifuged at 300 × g for 5 min to prevent loss of the loosely adherent cells; this step was not required for the highly adherent BMDM. The growth/differentiation medium was removed from BMDC- or BMDM-containing plates and replaced with DMEM (10% bovine calf serum, penicillin, streptomycin, and L-glutamine) supplemented with either 1 µg/ml LPS, 2 µg/ml PamC3Sk4, or 100 ng/ml TNF-α as “signal 1” priming stimuli to induce Nlrp3–dependent upregulation of pro–IL-1β and Nlrp3 expression. The cells were cultured with signal 1 for 4 h at 37°C. Plates with primed BMDC were again centrifuged at 300 × g for 5 min before further manipulation. For either BMDC or BMDM cultures, the priming medium was aspirated after 4 h and replaced with either Ca2+-containing balanced salt solution (BSS) (130 mM NaCl, 4 mM KCl, 1.5 mM CaCl2, 1 mM MgCl2, 25 mM Na HEPES, 5 mM d-glucose, 0.1% BSA [pH 7.4]), Ca2+-free BSS (130 mM NaCl, 4 mM KCl, 300 mM EGTA, 1 mLucose, 0.1% BSA [pH 7.4]), or high-K+ BSS (134 mM NaCl, 1.5 mM CaCl2, 1.5 mM MgCl2, 25 mM Na HEPES, 5 mM d-glucose, 0.1% BSA [pH 7.4]). BMDC and BMDM in Ca2+-containing medium were preincubated for 5 min at 37°C and then stimulated with 10 µg/mL nigericin, 5 mM ATP, 1 mM LLME, or other Ca2+-mobilizing agents for 30 min. Where indicated, signal 1–primed BMDC and BMDM were treated with 300 mM NAA for 15 s. The cell-free supernatant was then assayed for murine IL-1β by sandwich ELISA (R&D Systems) according to the manufacturer’s protocol. Western blot analysis of caspase-1 processing/release and IL-1β processing/release

Signal 1–primed BMDC in six-well plates were stimulated with nigericin, ATP, or other Ca2+-mobilizing agents at 37°C. After 30 min, extracellular medium was removed from each well and centrifuged at 10,000 × g for 15 s to pellet detached cells. The cell-free supernatants were then assayed for murine IL-1β by sandwich ELISA (R&D Systems) according to the manufacturer’s protocol.

ELISA analysis of IL-1β release

Signal 1–primed BMDC in 24-well plates were stimulated with nigericin, ATP, or other Ca2+-mobilizing agents at 37°C. After 30 min, extracellular medium was removed from each well and briefly centrifuged to pellet detached cells. The cell-free supernatants were then assayed for murine IL-1β by sandwich ELISA (R&D Systems) according to the manufacturer’s protocol.
Cell lysates and matching extracellular medium samples were subjected to SDS-PAGE and transferred to a polyvinylidene fluoride membrane for Western blot analysis. Primary Abs were used at the following concentrations: 1 μg/ml for caspase-1, 5 μg/ml for IL-1β, and 0.4 μg/ml for ASC. HRP-conjugated secondary Abs were used at a concentration of 0.13 μg/ml. Chemiluminescent images of Western blots were developed and saved using a FluorChem E image processor (Cell Biosciences).

Assay of ASC oligomerization using DSS-crosslinked detergent-insoluble lysate fractions

At stimulation, the extracellular medium from each well was removed, centrifuged at 10,000 × g for 15 s to pellet any detached BMDC, and the cell-free supernatant was aspirated. The adherent BMDC in each well were washed with 1 ml ice-cold PBS prior to preparation of whole-cell detergent lysates by addition of 150 μl lysis buffer (0.5% sodium deoxycholate, 0.1% SDS, 1% Igepal CA-650 in PBS [pH 7.4], plus protease inhibitor mixture), scraped with a rubber policeman to fully detach the cells, and incubated on ice for 5 min. This cell lysate was pooled from the retrieved whole-cell lysate, incubated for an additional 15 min on ice. The cell lysates were then separated into detergent-soluble and detergent-insoluble fractions by centrifugation at 15,000 × g for 5 min at 4°C. SDS sample buffer was added to detergent-soluble fractions for extraction at 100°C for 5 min. The detergent-insoluble lysate pellet was washed twice with 200 μl ice-cold PBS and then suspended in 200 μl PBS containing 20 mM DSS solution (in DMSO). The resuspended detergent-insoluble fractions were incubated with DSS for 30 min at room temperature, repelleted by centrifugation at 8000 × g for 15 min at room temperature, and the DSS supernatant solution was removed. The DSS-treated pellets were suspended in SDSPAGE buffer and extracted at 100°C for 5 min. The DSS-treated fractions were resolved by SDS-PAGE, transferred to polyvinylidene fluoride membrane, and analyzed by anti-ASC Western blotting.

Atomic absorption spectroscopy for measurement of K⁺ efflux

Signal 1–primed BMDC in 12-well plates (10⁵ cells/1 ml BS5/well) were stimulated for 30 min with 10 μM nigericin, ATP, LLME, or other Ca²⁺-mobilizing agents at 37°C. After 30 min, the extracellular medium was removed and centrifuged at 10,000 × g for 15 s to pellet detached cells. Adherent cells were cleared with 2 ml DSS-free BSS (5 mM Tris to chelate Ca²⁺ and quantify the minimum Ca²⁺-independent influx of fluo-4-AM in 1:1 proportion by volume), and 2.5 mM probenecid per well. After incubation at 37°C for 45 min, each well was briefly washed with PBS prior to the addition of a fresh 0.5-ml aliquot of BSS (either Ca²⁺-containing or Ca²⁺-free) supplemented with 2.5 mM probenecid. The plate was placed into the Synergy HT reader preheated to 37°C. Baseline fluorescence (485 nm excitation → 528 nm emission at 30-s intervals) was recorded for 10 min. Cells were then supplemented with 10 μM nigericin, 5 mM ATP, 1 mM LLME, or indicated concentrations of Ca²⁺-mobilizing agents for 30 min and changes in 485→528-em fluorescence were recorded at 30-s intervals. Where indicated, cells were treated with 300 nM thapsigargin in Ca²⁺-free BSS for 30 min to deplete ER Ca²⁺ stores prior to addition of nigericin or ATP. Assays were terminated by permeabilization of the cells with 1% Triton X-100 in the presence of excess CaCl₂ to quantify the maximum Ca²⁺-dependent fluorescence (Fₘₐₓ) of the fluo-4 in intact plasma membrane. The wells were then supplemented with 15 mM EGTA/50 mM Tris to chelate Ca²⁺ and quantify the minimum Ca²⁺-independent fluorescence of fluo-4 (Fₘₐₙ). The Fₘₐₓ and Fₘₐₙ values were used to calculate the cytosolic [Ca²⁺] of the intact cells as described by Tsien and colleagues (23).

Assay of cytotoxicity (LDH release)

Signal 1–primed BMDC in 24-well plates (5 × 10⁵ cells/0.5 ml BS5/well) were stimulated for 30 min with 10 μM nigericin or 1 mM LLME at 37°C. Extracellular medium was removed from each well and centrifuged at 10,000 × g for 15 s to pellet detached cells. The cell-free supernatant were then assayed for LDH enzyme activity using an LDH cytotoxicity detection kit (Roche) according to the manufacturer’s protocol. The released LDH was normalized to total LDH content measured in 1% Triton X-100-permeabilized samples of BMDC.

Data processing and analysis

All experiments were repeated 2–10 times with separate BMDC or BMDM preparations. Figures illustrating Western blot results are from representative experiments. Figures illustrating quantified changes in IL-1β secretion, intracellular K⁺ content, cytosolic [Ca²⁺], or pyroptotic propidium²+ fluorescence (these cells AM in 1:1 proportion by volume), and 2.5 mM probenecid per well. After incubation at 37°C for 45 min, each well was briefly washed with PBS prior to the addition of a fresh 0.5-ml aliquot of BSS (either Ca²⁺-containing or Ca²⁺-free) supplemented with 2.5 mM probenecid. The plate was placed into the Synergy HT reader preheated to 37°C. Baseline fluorescence (485 nm excitation → 528 nm emission at 30-s intervals) was recorded for 10 min. Cells were then supplemented with 10 μM nigericin, 5 mM ATP, 1 mM LLME, or indicated concentrations of Ca²⁺-mobilizing agents for 30 min and changes in 485→528-em fluorescence were recorded at 30-s intervals. Where indicated, cells were treated with 300 nM thapsigargin in Ca²⁺-free BSS for 30 min to deplete ER Ca²⁺ stores prior to addition of nigericin or ATP. Assays were terminated by permeabilization of the cells with 1% Triton X-100 in the presence of excess CaCl₂ to quantify the maximum Ca²⁺-dependent fluorescence (Fₘₐₓ) of the fluo-4 in intact plasma membrane. The wells were then supplemented with 15 mM EGTA/50 mM Tris to chelate Ca²⁺ and quantify the minimum Ca²⁺-independent fluorescence of fluo-4 (Fₘₐₙ). The Fₘₐₓ and Fₘₐₙ values were used to calculate the cytosolic [Ca²⁺] of the intact cells as described by Tsien and colleagues (23).

Assay of cytotoxicity (LDH release)

Signal 1–primed BMDC in 24-well plates (5 × 10⁵ cells/0.5 ml BS5/well) were stimulated for 30 min with 10 μM nigericin or 1 mM LLME at 37°C. Extracellular medium was removed from each well and centrifuged at 10,000 × g for 15 s to pellet detached cells. The cell-free supernatant were then assayed for LDH enzyme activity using an LDH cytotoxicity detection kit (Roche) according to the manufacturer’s protocol. The released LDH was normalized to total LDH content measured in 1% Triton X-100-permeabilized samples of BMDC.

Data processing and analysis

All experiments were repeated 2–10 times with separate BMDC or BMDM preparations. Figures illustrating Western blot results are from representative experiments. Figures illustrating quantified changes in IL-1β secretion, intracellular K⁺ content, cytosolic [Ca²⁺], or pyroptotic propidium²+ influx represent the means (±SE) from 2 to 10 independent experiments. Quantified data were statistically evaluated by one-way ANOVA with a Bonferroni posttest using Prism 3.0 software.

Results

Nigericin-induced increases in cytosolic [Ca²⁺] in LPS-primed BMDC occur downstream of the NLRP3 inflammasome/caspase-1/pyroptotic signaling cascade

Millimolar extracellular ATP gates the opening of plasma membrane P2X7 receptor nonselective cation channels that directly facilitate both efflux of cytosolic K⁺ and a massive influx of extracellular Ca²⁺ to cause a large increase in cytosolic [Ca²⁺] (19). In contrast, nigericin is a lipophilic ionophore that directly functions as a K⁺/H⁺ exchanger upon partitioning into the plasma membrane or intracellular organelles (Fig. 1A). Nigericin is highly selective for monovalent cations and does not directly facilitate transmembrane Ca²⁺ fluxes (20). Thus, any nigericin-induced increases in cytosolic [Ca²⁺] will reflect secondary changes in the activity of Ca²⁺ channels, nonselective ion channels, or Ca²⁺ homeostatic transporters due to altered [K⁺] or H⁺ concentration within the cytosol or organellar compartments. Although ATP and nigericin trigger efflux of cytosolic K⁺ by distinct mechanisms, both agonists induce similar magnitudes of caspase-1–dependent IL-1β release (Fig. 1B) in LPS-primed murine BMDC. The relative magnitudes of ATP-stimulated versus nigericin-stimulated IL-1β release can vary modestly between different primary BMDC cultures, with some showing equivalent responses to the two agonists (see Figs. 4C, 5G), others exhibiting slightly higher ATP efficacy (Fig. 2B; see Fig. 5C), and some showing lower ATP efficacy (Fig. 1B). This likely reflects modest differences in the expression levels of the ionotropic P2X7 receptors. We previously reported that accumulation of extracellular IL-1β by nigericin- and ATP-stimulated BMDC is maximal within 30 min (24). The responses are completely abrogated in Casp1/11⁻/⁻ (these cells
Ca^2+-INDEPENDENT NLRP3 INFLAMMASOME ACTIVATION

Nigericin-induced increases in cytosolic [Ca^{2+}] in LPS-primed BMDC occur downstream of the NLRP3 inflammasome/caspase-1/pyroptotic signaling cascade. (A) Diagram of changes in cation homeostasis occurring in myeloid cells in response to treatment with nigericin and ATP/P2X7. (B) IL-1β release from LPS-primed WT, Nlrp3^-/-, and Casp-1/11^-/- BMDC in response to 10 μM nigericin and 5 mM ATP was measured by ELISA. Data represent mean of two independent experiments. (C) LPS-primed WT, Nlrp3^-/-, and Casp-1/11^-/- BMDC were stimulated with 10 μM nigericin or 5 mM ATP for 30 min. BMDC were lysed with 10% nitric acid and lysates were analyzed by atomic absorption spectroscopy to measure cellular [K^+]. Data represent mean of three independent experiments. (D-F) WT, Nlrp3^-/-, or Casp-1/11^-/- BMDC were primed with LPS (1 μg/ml) for 4 h and loaded with 1 μg/ml fluo-4-AM for 30 min. Baseline readings were taken for 5 min and 10 μM nigericin (NG) or 5 mM ATP were added at t = 5 min. Cytosolic [Ca^{2+}] was determined by measuring fluo-4 fluorescence. Data represent a mean of two independent experiments. (G-I) LPS-primed WT, Nlrp3^-/-, and Casp-1/11^-/- BMDC were stimulated with 10 μM nigericin or 5 mM ATP for 30 min. Onset of pyroptosis was determined by measuring permeability of the cell membrane to propidium^2+. Baseline readings were taken for 5 min and nigericin or ATP were added at t = 5 min. Data represent a mean of two independent experiments.

are also deficient in caspase-11) and Nlrp3^-/- BMDC (Fig. 1B) or in WT BMDC stimulated in media with elevated extracellular [K^+] to prevent efflux of cytosolic [K^+] (24). Nigericin and ATP also induced robust decreases in cytosolic [K^+] in WT, Casp1/11^-/-, or Nlrp3^-/- cells (Fig. 1C). Thus, key steps in the upstream NLRP3 inflammasome signaling cascade that culminate in IL-1β release are maximally activated within this 30-min interval. To characterize how changes in cytosolic [Ca^{2+}] are temporally regulated during this critical phase of ATP- and nigericin-induced NLRP3 inflammasome activation, we used BMDC loaded with fluo-4 fluorescent Ca^{2+} sensor dye and assayed under experimental conditions (5 × 10^7 cells/0.5 ml test medium/well in 24-well plates at 37°C) identical to those used to measure IL-1β release. Stimulation of LPS-primed BMDC with 5 mM ATP induced an
∼20-fold increase in cytosolic [Ca\(^{2+}\)] from the basal level of 50–60 nM; the change in [Ca\(^{2+}\)] peaked within 3 min and then modestly decreased during the next 30 min to values 6- to 10-fold above basal (Fig. 1D). In contrast, cytosolic [Ca\(^{2+}\)] did not change during the first 10–12 min after nigericin stimulation and then gradually increased during the next 20 min to values only 3- to 4-fold above basal (Fig. 1D). Notably, ATP stimulated rapid and robust increases in cytosolic [Ca\(^{2+}\)] in Casp1/11\(^{-/-}\) BMDC (Fig. 1E) and Nlrc3\(^{-/-}\) BMDC (Fig. 1F), albeit with somewhat different kinetics. In contrast, the delayed and modest increase in cytosolic [Ca\(^{2+}\)] triggered by nigericin in WT cells was absent in Casp1/11\(^{-/-}\) or Nlrc3\(^{-/-}\) BMDC (Fig. 1D, 1E). These findings suggest that the nigericin-induced rise in [Ca\(^{2+}\)] observed in WT BMDC occurs downstream of NLRP3 inflammasome activation as a result of caspase-1–dependent changes in plasma membrane permeability.

The ability of caspase-1 to alter plasma membrane permeability as part of the proinflammatory cell death mechanism of pyroptosis is increasingly recognized as another readout of caspase-1 activity independent of its canonical action as an IL-1β converting enzyme (6). Cookson and colleagues (25, 26) have developed assays based on the uptake of small cationic DNA-intercalating dyes, such as ethidium\(^{+}\), to track these caspase-1–dependent changes in membrane permeability during pyroptotic progression in macrophages infected with *Salmonella* or *Anthrax*. We reasoned that the nigericin-induced increases in cytosolic [Ca\(^{2+}\)] may occur as a secondary consequence of the caspase-1–dependent increase in membrane permeability. We adapted the methods developed by Cookson and colleagues in an assay that measured the onset and progression of this permeability increase under the same experimental conditions used to monitor changes in cytosolic [Ca\(^{2+}\)] and IL-1β release. The assay tracks influx of the normally impermeant propidium\(^{2+}\) organic cation (\(M_r\) of 416 Da as free cation; \(M_r\) of 668 Da as propidium iodide salt) that undergoes a large increase in 540→620 em fluorescence upon intercalation with nuclear DNA. Nigericin initiated an influx of propidium\(^{2+}\) into WT BMDC after a 12- to 15-min delay following its addition; thereafter, the extent of propidium\(^{2+}\) uptake into the BMDC population rapidly increased, with ~80% of the cells accumulating the dye during the 30-min stimulation with nigericin (Fig. 1G). The delay phase and kinetics characterizing nigericin-induced propidium\(^{2+}\) influx were well correlated with the delay phase and kinetics of the nigericin-
triggered increase in cytosolic [Ca\(^{2+}\)] (Fig. 1D). Although influx of propidium\(^{3+}\) occurred earlier (by \(~6–8\) min) in response to ATP than to nigericin, the time course of the ATP-stimulated propidium\(^{3+}\) uptake (Fig. 1G) was markedly different from the ATP-induced Ca\(^{2+}\) increase response (Fig. 1D). The propidium\(^{3+}\) influx responses to both nigericin and ATP-gated P2X7 channels were absent in \textit{Casp1/11} \(^{-/-}\) (Fig. 1H) or \textit{Nlrp3} \(^{-/-}\) (Fig. 1I) BMDC and thus reflected induction of the pyroptotic membrane permeability transition. These studies demonstrate that the canonical NLRP3 activator nigericin does induce an increase in cytosolic [Ca\(^{2+}\)] in murine BMDC. However, this rise in [Ca\(^{2+}\)] occurs downstream of inflammasome activation as a secondary consequence of the pyroptotic membrane permeability transition (Fig. 1A). Enhanced Ca\(^{2+}\) influx may be a general response downstream of diverse caspase-1 inflammasome platforms. Vance and colleagues (27) reported that flagellin activation of NLRC4 inflammasomes in tissue-resident mouse macrophages triggered a caspase-1-dependent Ca\(^{2+}\) influx that preceded cytolysis and was required for phospholipase A2-dependent eicosanoid production; this was linked to a Ca\(^{2+}\)-dependent “eicosanoid storm” in vivo, leading to systemic shock and death of the mice.

**NLRP3 inflammasome signaling responses to K\(^{+}\) efflux agonists or lysosomal destabilization are dissociated from influx of extracellular Ca\(^{2+}\)**

That the nigericin-induced increases in cytosolic [Ca\(^{2+}\)] occur downstream of NLRP3-dependent caspase-1 activation argues against a necessary role for increased [Ca\(^{2+}\)] in the regulation of this inflammasome pathway in response to K\(^{+}\) efflux stimuli in the murine BMDC model. However, we directly tested the contribution of extracellular Ca\(^{2+}\) and Ca\(^{2+}\) influx to nigericin- and ATP-induced NLRP3 inflammasome signaling by comparison of BMDC incubated in standard Ca\(^{2+}\)-containing (1.5 mM CaCl\(_2\)) BSS versus Ca\(^{2+}\)-free BSS (no added CaCl\(_2\) plus supplementation with 0.3 mM EGTA). Removal of Ca\(^{2+}\) from the extracellular medium eliminated the increases in cytosolic [Ca\(^{2+}\)] triggered by nigericin or ATP (Fig. 2A). Despite the absence of detectable increases in cytosolic [Ca\(^{2+}\)], the IL-1β release responses to nigericin or ATP were not inhibited by the removal of extracellular Ca\(^{2+}\) (Fig. 2B, 2C). Similarly, the processing/release of caspase-1 and the formation of ASC oligomers in response to nigericin or ATP were not attenuated when BMDC were stimulated in Ca\(^{2+}\)-free medium (Fig. 2C). Notably, the delay phases characterizing induction of the propidium\(^{3+}\) permeability transition in nigericin- or ATP-stimulated cells were shortened in the absence of extracellular Ca\(^{2+}\) and the rates of propidium\(^{3+}\) influx were accelerated (Fig. 2D). As expected, both nigericin and ATP caused significant decreases in cellular [K\(^{+}\)] (Fig. 2E), but this response was similar in either the presence or absence of extracellular Ca\(^{2+}\) and measurable Ca\(^{2+}\) influx. These findings demonstrate that influx of extracellular Ca\(^{2+}\) and the resulting increase in cytosolic Ca\(^{2+}\) are not necessary signals for NLRP3 inflammasome activation in BMDC stimulated with agents that directly trigger rapid efflux of cytosolic K\(^{+}\). Brough et al. (11) reported the similar observation that Ca\(^{2+}\)-free medium did not inhibit, but rather increased, ATP-stimulated IL-1β release from isolated murine peritoneal macrophages.

We also tested whether changes in cytosolic [Ca\(^{2+}\)] are required for NLRP3 inflammasome activation by stimuli that induce lysosomal destabilization. Crystalline particulates or insoluble protein aggregates induce NLRP3 inflammasome assembly via a pathway involving phagocytosis of the particulates, phagosome maturation/fusion with lysosomes, and compromise of lysosome integrity (28, 29). Because the processes of particulate phagocytosis and phagolysosomal maturation/preservation may be modulated by perturbation of Ca\(^{2+}\) signaling, we used the soluble lysosomotropic agent LLME to induce rapid and synchronous lysosome disruption in LPS-primed BMDC. LLME enters cells via amino acid transporters, accumulates in lysosomes, and is then converted into membrane-disruptive polypeptide peptides (e.g., Leu\(_4\), Leu\(_6\)) via a reverse condensation reaction catalyzed by the cathepsin Cl/dipeptidyl peptidase I (30). It has been widely used as an NLRP3 activation stimulus in multiple inflammatory models (10). A 30-min incubation with 1 mM LLME induced robust release of IL-1β from WT, but not \textit{Casp1/11} \(^{-/-}\) or \textit{Nlrp3} \(^{-/-}\) BMDC (Fig. 3A).

Consistent with previous findings in murine macrophages (10), LLME stimulated equivalent K\(^{+}\) efflux from WT, \textit{Casp1/11} \(^{-/-}\) or \textit{Nlrp3} \(^{-/-}\) BMDC (Fig. 3B). Although the mechanism by which LLME or particulate lysosomal destabilizing stimuli elicit K\(^{+}\) efflux has not been defined, Zhong et al. (31) reported that aluminum hydroxide and silica induce activation of TRMP2 nonselective cation channels in murine macrophages as assayed by Ca\(^{2+}\) influx; there was also partial inhibition of IL-1β release in \textit{Trpm2} \(^{-/-}\) cells. Notably, LLME induced Ca\(^{2+}\) influx into BMDC after a 5-min lag period to result in a >10-fold sustained increase in cytosolic [Ca\(^{2+}\)] within 20 min (Fig. 3D). This increase was absent when BMDC were stimulated by LLME in Ca\(^{2+}\)-free BSS. Despite the absence of a detectable increase in cytosolic [Ca\(^{2+}\)] under these latter conditions, the IL-1β release response to LLME was not inhibited, but rather was potentiated, by removal of extracellular Ca\(^{2+}\) (Fig. 3C). LLME also stimulated propidium\(^{2+}\) influx after a 15-min lag period under control (1.5 mM extracellular Ca\(^{2+}\)) conditions (Fig. 3E); removal of extracellular Ca\(^{2+}\) modestly shortened the lag phase and increased the rate of propidium\(^{2+}\) influx. Thus, lysosome destabilization, similar to ATP gating of P2X7 channels, induces a Ca\(^{2+}\) influx that precedes, but is not required for, caspase-1–dependent IL-1β release or the propidium\(^{2+}\) influx response, which tracks with caspase-1 activation. Notably, the rates of LLME-stimulated Ca\(^{2+}\) influx were attenuated in \textit{Casp1/11} \(^{-/-}\) and \textit{Nlrp3} \(^{-/-}\) BMDC (Fig. 3F). This suggests that the net Ca\(^{2+}\) influx response induced by LLME in WT DC reflects contributions from both an inflammasome-independent cation permeability pathway (that also mediates K\(^{+}\) efflux) and an inflammasome/caspase-1–dependent change in permeability (that also mediates propidium\(^{2+}\) influx).

**NLRP3 inflammasome signaling responses to K\(^{+}\) efflux agonists are dissociated from release of thapsigargin-sensitive intracellular Ca\(^{2+}\) stores**

The absence of extracellular Ca\(^{2+}\) effectively eliminated the increases in cytosolic [Ca\(^{2+}\)] in response to nigericin or ATP but did not attenuate NLRP3 inflammasome activation in BMDC. However, several reports have suggested that mobilization of intracellular Ca\(^{2+}\) stores may be the critical regulatory signal. These studies have proposed that microdomains of the ER membrane system adjacent to mitochondria can release sufficient local Ca\(^{2+}\) to induce mitochondrial dysfunction leading to generation of reactive oxygen species and release of mitochondrial DNA into the cytosol. The mitochondria-derived reactive oxygen species and DNA then directly activate NLRP3 conformational changes to drive inflammasome assembly and signaling (32). To investigate the role of released ER Ca\(^{2+}\) stores in the activation of NLRP3 inflammasome signaling, we treated BMDC with thapsigargin, an inhibitor of the ER Ca\(^{2+}\) ATPase, for 30 min prior to stimulation with nigericin or ATP. Submicromolar thapsigargin inhibits all isoforms of the sarcoplasmic/ER calcium ATPases that actively maintain high intraluminal concentrations (0.3–1 mM) of free Ca\(^{2+}\) within the ER (33). Thapsigargin inhibition of sarcoplasmic/ER
calcium ATPase pump activity facilitates the rapid efflux of this stored Ca$^{2+}$ into the cytosol via as yet undefined “leak” channels. Ca$^{2+}$ released into the cytosol is rapidly transported to the extracellular compartment via the combined actions of the plasma membrane Ca$^{2+}$ ATPase pump and Na$^+$/Ca$^{2+}$ exchange transporters. However, the reduction in intraluminal [Ca$^{2+}$] in thapsigargin-treated cells also induces oligomerization of STIM family sensor proteins in the ER membrane, and the oligomerized STIM puncta activate conformational changes in Orai family store-operated Ca$^{2+}$ influx channels within juxtaposed domains of the plasma membrane (34). In the presence of extracellular Ca$^{2+}$, this STIM-dependent gating of Orai channels facilitates Ca$^{2+}$ influx to offset the loss of intracellular Ca$^{2+}$ and IL-1$\beta$ release was determined by ELISA. Data represent a mean of two independent experiments. (D) Cytosolic [Ca$^{2+}$] was determined as described in Fig. 1 in WT (D and F), Nlrp3$^{-/-}$, and Casp1$^{-/-}$ BMDC. Baseline readings were taken for 5 min and LLME was added at $t$ = 5 min. Data represent a mean of two independent experiments. (E) LLME-induced propidium$^+$ influx was measured in LPS-primed BMDC in the presence/absence of 1.5 mM extracellular [Ca$^{2+}$]. Baseline readings were taken for 5 min and LLME was added at $t$ = 5 min. Data represent mean of two independent experiments.

FIGURE 3. NLRP3 inflammasome signaling responses to lysosomal destabilization are dissociated from influx of extracellular Ca$^{2+}$. (A–C) LPS-primed BMDC were stimulated with 1 mM LLME for 30 min. (A) LPS-primed WT, Nlrp3$^{-/-}$, and Casp1$^{-/-}$ BMDC were stimulated with LLME and IL-1$\beta$ release was determined by ELISA. Data represent a mean of two independent experiments. (B) LPS-primed WT, Nlrp3$^{-/-}$, and Casp1$^{-/-}$ BMDC were stimulated with LLME, lysed with 10% nitric acid, and the lysates were analyzed by atomic absorption spectroscopy to measure cellular [K$^+$]. Data represent mean of three independent experiments. (C) LPS-primed WT BMDC were stimulated with LLME in the presence/absence of 1.5 mM extracellular [Ca$^{2+}$] and IL-1$\beta$ release was determined by ELISA. Data represent a mean of two independent experiments. (D) Cytosolic [Ca$^{2+}$] was determined as described in Fig. 1 in WT (D and F), Nlrp3$^{-/-}$, and Casp1$^{-/-}$ BMDC. Baseline readings were taken for 5 min and LLME was added at $t$ = 5 min. Data represent a mean of two independent experiments. (E) LPS-primed WT, Nlrp3$^{-/-}$, and Casp1$^{-/-}$ BMDC were stimulated with LLME in the presence/absence of 1.5 mM extracellular [Ca$^{2+}$]. Baseline readings were taken for 5 min and LLME was added at $t$ = 5 min. Data represent mean of two independent experiments.

Although the above findings indicate that increases in cytosolic [Ca$^{2+}$] are not required for nigericin- or ATP-stimulated NLRP3 activation in BMDC, it is possible that Ca$^{2+}$ may act as a modulatory signal by increasing the efficiency of productive NLRP3 inflammasome assembly. Thus far, LPS was used as the priming stimulus to upregulate

nigericin or ATP (Fig. 4B, 4C). Positive control experiments using cells treated with thapsigargin in Ca$^{2+}$-containing BSS verified the efficacy of thapsigargin as a Ca$^{2+}$-mobilizing agent in this BMDC model system (Fig. 4A, closed symbols). These findings demonstrate that release of ER Ca$^{2+}$ stores is also not a necessary signal for NLRP3 inflammasome activation in BMDC primed with TLR2 or TNF receptor agonists. Previously, Menu et al. (35) observed that micromolar concentrations of thapsigargin activate NLRP3 inflammasome signaling via induction of an ER stress response. In the above experiments we used a lower concentration of thapsigargin (300 nM) than in the Menu et al. study and did not observe induction of caspase-1–dependent pyroptosis or significant IL-1$\beta$ release by BMDC in response to treatment with thapsigargin alone (Fig. 4B, 4C). NLRP3 inflammasome signaling responses to K$^+$ efflux agonists are dissociated from changes in cytosolic [Ca$^{2+}$] in BMDC primed with TLR2 or TNF receptor agonists
expression of NLRP3 and pro–IL-1β. Given the ability of TLR4 to drive both MyD88- and TRIF-dependent signaling pathways, LPS is a particularly strong activator of the NF-κB–based transcription of these proinflammatory gene products, and we have previously reported that NLRP3 is expressed at high levels in LPS-primed BMDC (24). With high levels of expression of inflammasome components, potential modulatory effects of elevated cytosolic [Ca\textsuperscript{2+}] on NLRP3 activation or inflammasome complex assembly may be negligible during stimulation with potent signal 2 agonists such as ATP or nigericin. To test for more subtle modulatory effects of cytosolic [Ca\textsuperscript{2+}] on NLRP3 inflammasome activation, we used less efficacious (relative to LPS) signal 1 priming stimuli, including the synthetic TLR2 agonist Pam3CSK4 and the proinflammatory cytokine TNF-α. Both nigericin and ATP triggered increases in cytosolic [Ca\textsuperscript{2+}] in BMDC primed with Pam3CSK4 (Fig. 5A) or TNF-α (Fig. 5E) that were similar in kinetics to those observed in the LPS-primed cells (Fig. 1D). Control experiments verified that, as expected, nigericin induced similar magnitudes of K\textsuperscript{+} efflux in Pam3CSK4- and TNF-α–primed cells as in unprimed or LPS-primed BMDC. Importantly, the nigericin-induced increases in Ca\textsuperscript{2+} influx occurred after a 10- to 15-min delay and correlated with the onset of pyroptotic propidium\textsuperscript{2+} influx in both the Pam3CSK4–primed (Fig. 5B) and TNF-α–primed BMDC (Fig. 5F). Consistent with the reduced efficacy of these signal 1 stimuli, the magnitudes of nigericin- or ATP-stimulated IL-1β production in Pam3CSK4–primed cells (Fig. 5C: ∼12–20 ng/ml/30 min in Ca\textsuperscript{2+}-containing saline), and especially in TNF-α–primed cells (Fig. 5G: ∼1.5 ng/ml/30 min), were lower than in LPS-primed cells (Figs. 1B, 2B, 3C: 15–25 ng/ml/30 min). Similarly, the percentages of BMDC that accumulated propidium\textsuperscript{2+} after 30 min of nigericin or ATP stimulation in Ca\textsuperscript{2+}-containing saline were lower with Pam3CSK4 priming (Fig. 5B: 50% with both agonists) and TNF-α priming (Fig. 5F: 45% with nigericin and 15% with ATP) than with LPS priming (Figs. 1G, 2D: 60–80% with both agonists). The absence of extracellular Ca\textsuperscript{2+} did not inhibit the pyroptotic propidium\textsuperscript{2+} influx (Fig. 5B, 5F) or IL-1β release (Fig. 5C, 5G) responses to nigericin or ATP in BMDC primed with Pam3CSK4 or TNF-α. Rather, as observed in LPS-primed BMDC, removal of extracellular Ca\textsuperscript{2+} from the Pam3CSK4- or TNF-α–primed BMDC during stimulation shortened the delay phases and increased the rates of the propidium\textsuperscript{2+} uptake responses. In Ca\textsuperscript{2+}-free medium, there was a trend (that did not reach statistical significance) for ATP, but not nigericin, to stimulate more IL-1β release in the TNF-α–primed cells (Fig. 5G). Interestingly, thapsigargin treatment (in Ca\textsuperscript{2+}-free saline) produced a modest (20–33%) decrease in the IL-1β release responses to ATP, but not nigericin, in the Pam3CSK4- and TNF-α–primed BMDC (Fig. 5D, 5H). Taken together, the experiments with Pam3CSK4- or TNF-α–primed BMDC also argue against a necessary role for increased Ca\textsuperscript{2+} influx and/or mobilization of intracellular Ca\textsuperscript{2+} stores in the activation of NLRP3 inflammasome signaling by K\textsuperscript{+} efflux agonists.

**NLRP3 inflammasome signaling responses to K\textsuperscript{+} efflux are dissociated from changes in cytosolic [Ca\textsuperscript{2+}] in murine macrophages**

Most studies implicating roles for increased cytosolic [Ca\textsuperscript{2+}] in activation of NLRP3 inflammasome signaling have been performed in murine or human macrophages (11–15). Although the key elements of NLRP3 inflammasome composition and function are conserved in macrophage and DC models, some differences in modulatory signals have been reported. For example, phosphorylation of ASC by Syk markedly potentiates NLRP3 inflammasome activation by nigericin in BMDM and peritoneal macrophages, but not BMDC (36). Therefore, we investigated the potential contribution of extracellular Ca\textsuperscript{2+} and ER Ca\textsuperscript{2+} stores to NLRP3 inflammasome activation in BMDM. As in BMDC, nigericin and ATP induced increases in cytosolic [Ca\textsuperscript{2+}] with distinctive time courses in BMDM (Fig. 6A). ATP triggered a rapid 20-fold increase from the 50 nM basal [Ca\textsuperscript{2+}] in the macrophages that peaked at 5 min poststimulation. However, the subsequent decrease was more rapid and robust than that observed in the BMDC (Fig. 1D). As in DC, the ATP-induced Ca\textsuperscript{2+} influx preceded the induction of propidium\textsuperscript{2+} influx by ∼8 min in the...
BMDM (Fig. 6B). The nigericin-induced increase in macrophage 
[Ca2+] was defined by a similar 10- to 12-min delay phase (Fig. 5A) as observed in the DC (Fig. 1D). This was followed by a slow rate of increase that plateaued at 200–300 nM (Fig. 6A); the delayed increase in [Ca2+] was also correlated with the onset of pyroptotic propidium2+ influx in the BMDM (Fig. 6B). As in BMDC, removal of extracellular Ca2+ did not suppress either the kinetics or the magnitudes of the propidium2+ influx responses to nigericin and ATP in BMDM (Fig. 6B). Similarly, stimulation of macrophages with nigericin or ATP in Ca2+-free saline did not inhibit, but rather had no effect (nigericin) or modestly enhanced (ATP), the IL-1β release responses (Fig. 6C). The combined removal of extracellular Ca2+ and depletion of ER Ca2+ stores by thapsigargin treatment in BMDM did not significantly change IL-1β release in response to either agonist (Fig. 6D). These findings indicate that increased cytosolic [Ca2+] is not a necessary signal for NLRP3 inflammasome activation in murine BMDM.

Increased cytosolic [Ca2+] induced by Ca2+ ionophore or Ca2+-mobilizing GPCR is not a sufficient signal for NLRP3 inflammasome activation

Several studies have reported that stimulation of myeloid cells with agonists for certain Gq or Gi-coupled receptors induces NLRP3 inflammasome activation via phospholipase C (PLC)–mediated generation of inositol trisphosphate (IP3) and release of ER Ca2+ stores (14–16). We compared the efficacy of various Ca2+-mobilizing agonists versus the K+ efflux agonists in the activation of NLRP3 inflammasome signaling by stimulating LPS-primed BMDC under the same test conditions (30 min in 1.5 mM CaCl2-containing saline) routinely used for induction by nigericin or P2X7 channel gating. The Ca2+-mobilizing agonists included 1) ionomycin, a Ca2+ ionophore that stimulates both influx of extracellular Ca2+ and release of ER Ca2+ stores (37); 2) R568, a synthetic agonist of the Gq-coupled CaSR reported to induce robust NLRP3 inflammasome activation in murine and human macrophages via the PLC/IP3-gated ER Ca2+ mobilization pathway (14, 15); 3) ATP or UTP, which at submillimolar concentrations activate the Gq/PLC-coupled P2Y2 nucleotide receptors highly expressed in all myeloid leukocyte subtypes (38); and 4) fMLF, a synthetic agonist of the Gi/PLC-coupled formyl peptide receptors (FPR) that facilitate chemotraction of myeloid leukocytes to local accumulations of bacteria-derived formylated proteins (39).

As expected, ionomycin mimicked the ability of ATP-gated P2X7 channels to induce a large and immediate increase in cytosolic [Ca2+] that was sustained for up to 30 min (Fig. 7A). Despite this robust elevation in cytosolic [Ca2+], the 30-min treatment with ionomycin did not elicit statistically significant release of IL-1β as indicated by ELISA (Fig. 7B) or Western blot for the mature 17-kDa cytokine (Fig. 7G). These observations are consistent with Brough et al. (11) and Murakami et al. (13) who reported a similar inability of ionomycin to activate caspase-1 and release of mature IL-1β in murine macrophages. Ionomycin also did not trigger rapid pyroptotic signaling in the
BMDC as demonstrated by the lack of propidium $^{2+}$ influx throughout the ionomycin exposure (Fig. 7E). Ionomycin did induce a modest 25–30% reduction in cellular K$^+$ content (Fig. 7F) that was independent of NLRP3 or caspase-1 expression (Supplemental Fig. 1A). Brough et al. (11) described an ionomycin-induced decrease in cell viability (release of 64% total LDH) in murine macrophages, which would necessarily be correlated with a decrease in total K$^+$ content. We observed much less ionomycin-induced cell death (release of 18–20% total LDH within 60 min) in either WT or $\text{Casp}1/1/2/2$ BMDC (Supplemental Fig. 1B), which contrasted with the 70% LDH release in ATP-treated WT cells and no LDH release in ATP-treated $\text{Caspl/11}^{+/−}$ cells. Thus, the 25–30% decrease in [K$^+$] induced by ionomycin reflects both cell death–dependent and cell death–independent components. The magnitude of this [K$^+$] decrease was significantly smaller than that triggered by nigericin or P2X7 channel gating (Fig. 7F). Notably, this decrease was not sufficient for robust inflammasome assembly as indicated by the barely detectable accumulation of ASC oligomers (Fig. 7G). The findings further suggest that cytosolic [K$^+$] must decrease below a threshold value to entrain the signaling pathways required for conformational activation of NLRP3.

All of the tested GPCR agonists triggered an immediate 4-fold increase in cytosolic [Ca$^{2+}$] that peaked within 60 s and then rapidly decreased during the next few minutes (Fig. 7C). The fMLF-, UTP-, or 100 $\mu$M ATP-triggered Ca$^{2+}$ transients decayed to the basal level within 5–7 min. In contrast, the R568-induced peak in cytosolic [Ca$^{2+}$] was followed by a sustained 3-fold elevation in cytosolic [Ca$^{2+}$] during the 30-min test period (Fig. 7C). No statistically significant release of IL-1β was observed in

**FIGURE 6.** NLRP3 inflammasome signaling responses to K$^+$ efflux agonists are dissociated from changes in cytosolic [Ca$^{2+}$] in murine macrophages. (A) Murine BMDM were primed with LPS (1 $\mu$g/ml) for 4 h. Baseline readings were taken for 5 min and 10 $\mu$M nigericin (NG) or 5 mM ATP was added at $t = 5$ min. Cytosolic [Ca$^{2+}$] was determined as described in Fig. 1. Data represent a mean of two independent experiments. (B and C) LPS-primed BMDM were treated for 30 min with 10 $\mu$M nigericin or 5 mM ATP in the presence/absence of 1.5 mM extracellular [Ca$^{2+}$]. (B) Ionomycin did induce a modest 25–30% reduction in cellular K$^+$ content (Fig. 7F) that was independent of NLRP3 or caspase-1 expression (Supplemental Fig. 1A). Brough et al. (11) described an ionomycin-induced decrease in cell viability (release of 64% total LDH) in murine macrophages, which would necessarily be correlated with a decrease in total K$^+$ content. We observed much less ionomycin-induced cell death (release of 18–20% total LDH within 60 min) in either WT or $\text{Casp}1/1/2/2$ BMDC (Supplemental Fig. 1B), which contrasted with the 70% LDH release in ATP-treated WT cells and no LDH release in ATP-treated $\text{Caspl/11}^{+/−}$ cells. Thus, the 25–30% decrease in [K$^+$] induced by ionomycin reflects both cell death–dependent and cell death–independent components. The magnitude of this [K$^+$] decrease was significantly smaller than that triggered by nigericin or P2X7 channel gating (Fig. 7F). Notably, this decrease was not sufficient for robust inflammasome assembly as indicated by the barely detectable accumulation of ASC oligomers (Fig. 7G). The findings further suggest that cytosolic [K$^+$] must decrease below a threshold value to entrain the signaling pathways required for conformational activation of NLRP3.

All of the tested GPCR agonists triggered an immediate 4-fold increase in cytosolic [Ca$^{2+}$] that peaked within 60 s and then rapidly decreased during the next few minutes (Fig. 7C). The fMLF-, UTP-, or 100 $\mu$M ATP-triggered Ca$^{2+}$ transients decayed to the basal level within 5–7 min. In contrast, the R568-induced peak in cytosolic [Ca$^{2+}$] was followed by a sustained 3-fold elevation in cytosolic [Ca$^{2+}$] during the 30-min test period (Fig. 7C). No statistically significant release of IL-1β was observed in
extracellular medium fraction (ECM) was probed for mature caspase-1 and IL-1.

lysate fraction (Lys) was probed for procaspase-1 and pro–IL-1 in independent experiments. (B) Cytosolic [Ca^{2+}] was determined as described in Fig. 1. Baseline readings were taken for 5 min and 5 mM ATP or 3 mM ionomycin was added at t = 5 min. Data represent a mean of two independent experiments. (C) LPS-primed BMDC were treated with 30 μM R568, 1 mM ATP, 1 μM iMLF, or 0.1 mM UTP for 30 min. Cytosolic calcium levels were determined as described in Fig. 1. Baseline readings were taken for 5 min and GPCR agonists were added at t = 5 min. Data are representative of two independent experiments. (D) LPS-primed BMDC were treated with calcium-mobilizing agents for 30 min. IL-1β release was measured by ELISA. Data represent mean of five independent experiments. (E) LPS-primed BMDC were treated with 10 μM nigericin, 5 mM ATP, or 3 mM ionomycin. Onset of pyroptosis was determined by measuring permeability of the cell membrane to propidium 2+. Baseline readings were taken for 5 min and nigericin, ATP, or ionomycin was added at t = 5 min. Data represent a mean of two independent experiments. (F) LPS-primed BMDC were treated with 10 μM nigericin, 5 mM ATP, or 3 mM ionomycin. Cells were lysed with 10% nitric acid and lysates were analyzed by atomic absorption spectroscopy to measure cellular [K^{+}]. Data represent mean of eight independent experiments. (G) LPS-primed BMDC were stimulated with 10 μM nigericin, 5 mM ATP, 3 mM ionomycin, or 30 μM R568 for 30 min. Soluble lysate fraction (Lys) was probed for procaspase-1 and pro–IL-1β, insoluble lysate pellet was crosslinked with DSS and probed for oligomerized ASC, and extracellular medium fraction (ECM) was probed for mature caspase-1 and IL-1β.

BMDC stimulated for 30 min with iMLF, UTP, 100 μM ATP, or R568 (Fig. 7D). Similarly, R568 did not induce detectable accumulation of IL-1β or caspase-1 p20 subunits in the extracellular medium (Fig. 7G) and elicited only weak accumulation of intracellular ASC oligomers (Fig. 7G). These data demonstrate that agonists for Ca^{2+}-mobilizing GPCR do not mimic the ability of K^{+} efflux agonists to elicit rapid and robust NLRP3 inflammasome activation in the murine BMDC model.

**Suppression of nigericin-stimulated NLRP3 inflammasome signaling by BAPTA and 2-APB can be dissociated from perturbation of Ca^{2+} signaling**

Some support implicating Ca^{2+} signaling in NLRP3 inflammasome activation is based on observations that BAPTA, a strong Ca^{2+} chelator and buffer of cytosolic Ca^{2+}, and 2-APB, an inhibitor of IP_{3}-gated Ca^{2+} release channels and store-operated calcium entry channels, strongly suppress IL-1β release in response to canonical NLRP3 activators (11–14). Our finding that removal of extracellular Ca^{2+} (with or without thapsigargin treatment) eliminates the increases in cytosolic [Ca^{2+}], but not the IL-1β release, stimulated by nigericin or ATP (Figs. 2, 4), is possible that the inhibitory actions of BAPTA and 2-APB on NLRP3 signaling may also be dissociated from effects on Ca^{2+} signaling. We observed that loading LPS-primed BMDC with BAPTA markedly attenuated multiple readouts of nigericin-stimulated NLRP3 inflammasome signaling, including total IL-1β release (Fig. 8A), extracellular accumulation of p20 caspase-1 subunit and p17 mature IL-1β (Fig. 8D), formation of ASC oligomers (Fig. 8D), and induction of pyroptotic propidium 2+ influx (Fig. 8C). Importantly, note that all assays were performed in the absence of extracellular Ca^{2+}, which effectively eliminates any nigericin-induced increase in cytosolic [Ca^{2+}] (Fig. 2A). In contrast, the nigericin-stimulated influx of cytosolic K^{+} was not inhibited in the BAPTA-loaded cells (Fig. 8B). We verified the efficacy of BAPTA loading and its ability to buffer the delayed decrease in cytosolic [Ca^{2+}] induced by nigericin stimulation when BMDC were incubated in 1.5 mM Ca^{2+}-containing basal saline (Supplemental Fig. 2). These findings demonstrate that cytosolic BAPTA loading can inhibit IL-1β processing and release independently of its function as a Ca^{2+} chelator and downstream of the necessary NLRP3-activating K^{+} efflux signal.

Previous studies have demonstrated that 2-APB strongly inhibits NLRP3 inflammasome activation in response to nigericin and other stimuli. This suppression has been ascribed to the extensively characterized actions of this reagent as an inhibitor of both IP_{3}-gated Ca^{2+} release channels in the ER and store-operated Ca^{2+}...
influx channels in the plasma membrane (13, 14). An expectation of this proposed mechanism is that the ability of 2-APB to inhibit nigericin-stimulated IL-1β release and pyroptosis should be highly correlated with suppression of Ca²⁺ mobilization and influx. We tested the effects of 2-APB on multiple indices of nigericin-activated NLRP3 inflammasome signaling in LPS-primed BMDC incubated in CaCl₂-containing medium. Consistent with previous findings (13, 14), we observed that 2-APB completely suppressed total IL-1β release (Fig. 9A), extracellular accumulation of p20 caspase-1 subunit and p17 mature IL-1β (Fig. 9C), formation of ASC oligomers (Fig. 9C), and induction of propidium²⁺ influx (Fig. 9D) in response to nigericin. In contrast, robust K⁺ efflux responses were observed in nigericin-treated BMDC in the absence or presence of 2-APB (Fig. 9B). Despite the complete suppression of these multiple readouts of NLRP3 signaling and the suppression of Ca²⁺ signaling, 2-APB by itself triggered large increases in cytosolic [Ca²⁺] and potentiated nigericin-induced Ca²⁺ influx in LPS-primed murine DC.

**Discussion**

Perturbation of intracellular ion homeostasis is a major cellular stress signal for activation of NLRP3 inflammasome signaling. However, the relative contributions of decreased cytosolic [K⁺] versus increased cytosolic [Ca²⁺] remain disputed and incompletely defined. This study provides three major findings relevant to this unresolved area of NLRP3 regulation. First, increased cytosolic [Ca²⁺] is neither a necessary nor sufficient signal for the NLRP3 inflammasome cascade induced in murine DC and macrophages during activation by endogenous ATP-gated P2X7 receptor channels, the bacterial ionophore nigericin, or LLME-induced lysosomal disruption. These stimuli are widely used as highly efficacious inducers of NLPR3 inflammasome assembly in murine and human myeloid leukocyte models. Second, agonists for three Ca²⁺-mobilizing GPCR expressed in murine myeloid leukocytes (FPR, P2Y2 purinergic receptor, CaSR) were ineffective as robust activators of NLRP3 signaling when directly compared with the K⁺ efflux agonists under identical experimental conditions. Third, BAPTA and 2-APB, widely used reagents for disruption of Ca²⁺-dependent signaling pathways, strongly suppress nigericin-induced NLRP3 inflammasome signaling via mechanisms dissociated from their canonical or expected effects on Ca²⁺ homeostasis.
We assessed the possible roles for influx of extracellular Ca\(^{2+}\) and/or mobilization of ER Ca\(^{2+}\) stores on multiple steps in the serial NLRP3 signaling pathway, including efflux of cytosolic K\(^{+}\), accumulation of stable ASC oligomers, production of active caspase-1, processing and release of mature IL-1\(\beta\), and induction of pyroptotic changes in plasma membrane permeability. Direct measurements using cells loaded with fluo-4 Ca\(^{2+}\) sensor dye verified the absence of cytosolic [Ca\(^{2+}\)] increases during manipulations designed to eliminate Ca\(^{2+}\) influx and mobilization in the ATP- or nigericin-treated cells. Recent reports have indicated that NLRP3 activation triggers the rapid assembly of prion-like ASC aggregates that comprise the critical and essentially irreversible step in coupling conformational changes in the NLRP3 stress sensor to activation of the caspase-1 effector enzyme (4, 5). Our findings indicate that ATP and nigericin induce equivalent accumulation of detergent-insoluble ASC aggregates regardless of the presence or absence of increases in cytosolic [Ca\(^{2+}\)]. Although our data indicate that Ca\(^{2+}\) is not a critical second messenger for the very proximal steps of the NLRP3 signaling cascade, changes in cytosolic [Ca\(^{2+}\)] may modulate reactions downstream of the NLRP3-dependent assembly of the ASC oligomeric platforms for caspase-1 activation, particularly the several nonclassical export pathways for release of mature IL-1\(\beta\) and caspase-1 itself. We previously reported that increases in cytosolic [Ca\(^{2+}\)] do not affect caspase-1–dependent processing of pro–IL-1\(\beta\) but can potentiate the release of processed mature

**FIGURE 9.** Suppression of nigericin-stimulated NLRP3 inflammasome signaling by 2-APB can be dissociated from perturbation of Ca\(^{2+}\) signaling. (A–C) LPS-primed BMDC were incubated with 100 \(\mu\)M 2-APB for 20 min prior to treatment with 10 \(\mu\)M nigericin for 30 min in the presence of 1.5 mM extracellular [Ca\(^{2+}\)]. (A) IL-1\(\beta\) release was measured by ELISA. Data represent a mean of three independent experiments. (B) Efflux of cellular K\(^{+}\) was measured by atomic absorption spectroscopy. Data represent a mean of three independent experiments. (C) Soluble lysate fraction (Lys) was probed for procaspase-1 and pro–IL-1\(\beta\), insoluble lysate pellet was crosslinked with DSS and probed for oligomerized ASC, and extracellular medium fraction (ECM) was probed for mature caspase-1 and IL-1\(\beta\). (D) LPS-primed BMDC were preincubated with 100 \(\mu\)M 2-APB for 20 min in the presence of 1.5 mM extracellular [Ca\(^{2+}\)] and onset of pyroptosis in response to nigericin was assayed by measuring permeability of cells to propidium\(\text{II}^{+}\). Baseline readings were taken for 5 min and 10 \(\mu\)M nigericin was added at \(t = 5\) min. (E) Cytosolic [Ca\(^{2+}\)] in LPS-primed BMDC was measured using fluo-4 fluorescence in the presence of 1.5 mM extracellular [Ca\(^{2+}\)]. Baseline readings were taken for 5 min. Cells were treated with 100 \(\mu\)M 2-APB at \(t = 5\) min (double arrow) and 10 \(\mu\)M nigericin (single arrow) at \(t = 15\) min. Data are representative of two independent experiments.
IL-1β in some, but not all, cell models (40, 41). Depending on cell type and activation stimulus, IL-1β can be released in different proportions via three vesicular and one nonvesicular mechanism (42). The vesicular mechanisms include 1) IL-1β trapped within plasma membrane-derived microvesicles that bleb and scission from the cell surface, 2) IL-1β packaged within exosomes contained in multivesicular endosomes that subsequently fuse with the cell surface membrane, and 3) exocytosis of IL-1β that has been internalized within secretory autophagolysosomes. The nonvesicular pathway is via regulated cell lysis as a consequence of caspase-1–driven pyroptosis. In our LPS-primed BMDC and BMDM models, the magnitudes of IL-1β release induced by nigericin or ATP were largely equivalent in the absence or presence of extracellular Ca2+ or in the absence or presence of thapsigargin treatment to deplete ER Ca2+ stores. We also measured propidium iodide influx as an index of the caspase-1–regulated transition in plasma membrane permeability that accompanies pyroptotic cell lysis (43). This parameter provides a readout of caspase-1 activation kinetics independent of IL-1β release by the vesicular mechanisms. Notably, DC and macrophages stimulated with nigericin or ATP in the absence or presence of extracellular Ca2+ exhibited similar onset and rates of propidium iodide influx. This indicates that the Ca2+-independent pathway predominantly mediates the nonclassical export of IL-1β following rapid inflammasome activation by K+ efflux agonists. However, in other inflammasome models with slower progression to pyroptosis, Ca2+-dependent vesicular IL-1β release may be more important. Differential roles for local mobilization of ER Ca2+ stores in downstream IL-1β release, rather than upstream inflammasome activation, may underlie the modest decreases we observed in the IL-1β release responses to ATP, but not nigericin, in the Pam3CSK4- and TNF-α–primed BMDC. 

Agonists for some, but not all, Ca2+-mobilizing GPCR expressed in myeloid leukocytes can induce the release of IL-1β via an NLRP3-dependent mechanism (14, 15). Rossol et al. (15) noted that NLRP3 in human and murine monocytes was a target for CaSR and GPRC6A activated by divalent/trivalent inorganic cations. However, those studies indicated that accumulation of extracellular IL-1β was a delayed response with little cytokine release occurring during the initial 3 h and maximal release requiring >8 h of CaSR activation (15). Lee et al. (14) described more rapid (within 30–50 min) CaSR-induced activation of NLRP3 signaling in murine BMDM and also used the organic calcimimetic agonist R568 to induce CaSR-dependent NLRP3 inflammasomes. We compared the relative efficacies of R568 and the K+ efflux agonists to drive rapid NLRP3 inflammasome activation in our LPS-primed BMDC experimental model. Although R568 rapidly induced a sustained increase in cytosolic Ca2+ during a 30-min test period, this resulted in only very weak accumulation of ASC oligomers that was insufficient to drive significant caspase-1 activation or IL-1β release. Different GPCR have varying rates of desensitization. For example, P2Y2 purinergic receptors desensitize within minutes after activation by the metabolically labile ATP/UTP agonists (44). In contrast, the canonical role of the CaSR is to respond to slowly developing increases in serum extracellular Ca2+ for regulation of parathyroid hormone secretion. The receptor is thus remarkably resistant to desensitization and retains the ability to signal via the GPCR cascade with sustained elevation of cytosolic Ca2+ for hours after exposure to agonistic stimuli (45). Sustained elevation of cytosolic Ca2+ during prolonged CaSR activation may induce sufficient activity of Ca2+-sensitive K+ channels to decrease cytosolic K+ to the threshold level required for stable assembly of NLRP3/ASC signaling platforms (46). At the single cell level, the assembly of such stable platforms likely comprises an all-or-none response to coincident combinations of critical regulatory signals, such as decreased cytosolic [K+] as well as the ubiquitination or phosphorylation states of NLRP3 and ASC (36, 47, 48). It would be relevant to test whether sustained stimulation of DC or macrophages with different slowly desensitizing GPCR gradually increases the number of cells with perinuclear ASC specks and accumulation of active caspase-1.

The Ca2+ chelator BAPTA has been used to implicate increased cytosolic [Ca2+] as a necessary signal for NLRP3 inflammasome activation in response to various stimuli, including nigericin and extracellular ATP (11–14). Although our studies confirmed the ability of BAPTA loading to markedly attenuate nigericin-induced NLRP3 signaling in LPS-primed BMDC, this inhibitory action was observed under Ca2+-free stimulation conditions that prevent nigericin-elicited changes in cytosolic [Ca2+]. BAPTA chelates other trace divalent cations, including Zn2+, Fe2+, and Cu2+, that can function as key cofactors for many enzymes, including some implicated in inflammasome regulation. Several reports have implicated roles for intracellular Zn2+ in modulation of NLRP3 inflammasome signaling (49). One possible specific link is the zinc-dependent metalloprotease, BRCC3, which acts as a K63-specific deubiquitinase and critical regulator of NLRP3. Py et al. (50) found that BRCC3 promotes NLRP3 inflammasome activation by deubiquitinating the LRR domain of NLRP3. In addition to ubiquitination, other studies have implicated the phosphorylation status of inflammasome components as critical to the efficient assembly of NLRP3/ASC platforms. Martin et al. (47) identified the PP2A phosphatase as a key target for signal 2 stimuli, including ATP and nigericin, which acted to recruit PP2A to complexes of IKKα and ASC. The recruited PP2A reverses association of IKKα with ASC and thus licenses ASC for interaction with NLRP3. Notably, BAPTA loading attenuated the recruitment of PP2A in response to ATP or nigericin (47). Previous studies have identified complex roles for cytosolic Zn2+ in regulation of inflammasome activity (51, 52). Finally, Furuta et al. (17) demonstrated that BAPTA exerts a potent microtubule-depolymerizing activity that is independent of its ability to chelate Ca2+. This is relevant because Misawa et al. (53) have described a microtubule-mediated spatial arrangement of mitochondria in close apposition to the ER that is necessary for optimal NLRP3 inflammasome activation. The ability of BAPTA to promote microtubule depolymerization might interfere with this organelle juxtaposition and thus attenuate interaction between ASC and NLRP3. Taken together, the combined effects of BAPTA on Zn2+-dependent signaling enzymes and/or microtubule dynamics may underlie its inhibitory actions on NLRP3 signaling independently of its canonical actions on Ca2+ signaling.

Additional support for the involvement of Ca2+ signaling in NLRP3 activation has come from experiments using 2-APB as an inhibitor of both ER Ca2+ release via IP3 receptor channels and influx of extracellular Ca2+ via the Orai family Ca2+ release–activated Ca2+ channels (13, 14). We confirmed previously reported observations that pretreatment of LPS-primed myeloid cells with 2-APB prior to stimulation by nigericin (or ATP) completely suppresses all indices of the activated NLRP3 signaling cascade. Importantly, our experiments also demonstrated that 2-APB inhibits the ability of nigericin to induce the pyroptotic propidium iodide influx as an alternative readout of caspase-1 activation. However, we were unable to correlate these robust inhibitory effects of 2-APB on NLRP3 signaling with the canonical inhibitory actions of 2-APB on elevation of cytosolic [Ca2+] and Ca2+ signaling. Rather, 2-APB per se caused increases in cytosolic [Ca2+] in LPS-primed DC and also facilitated massive influx of Ca2+.
during subsequent stimulation by nigericin (Fig. 9E). Although 2-APB does indeed potently block Ca\(^{2+}\) influx via the Orai1 and Orai2 subtypes of Ca\(^{2+}\) release–activated Ca\(^{2+}\) channels, it has the opposite effect on the Orai3 family member. Several groups have described the ability of 2-APB to allosterically stabilize the open-gated conformation of Orai3 channels and change their permeability properties into nonselective cation channels that facilitate fluxes of Ca\(^{2+}\) and monovalent cations (54–59). The ability of 2-APB to stimulate increased cytosolic [Ca\(^{2+}\)] in our LPS-primed BMDC model suggests that these cells express significant levels of Orai3 channels, and this is supported by our preliminary Western blot analyses of BMDC and BMDM. Regardless of how 2-APB induces Ca\(^{2+}\) influx, our observations indicate that the suppression of NLRP3 inflammasome signaling in 2-APB–treated BMDC cannot be ascribed to direct inhibition of Ca\(^{2+}\) signaling. 2-APB does not inhibit the inflammasome signaling cascades initiated by AIM2 (14). Given that AIM2, similar to NLRP3, regulates an ASC-dependent inflammasome, this suggests that 2-APB targets NLRP3 function rather than ASC. Additional studies are required to define the underlying pharmacological mechanisms by which 2-APB may inhibit NLRP3 upstream of ASC oligomerization.

In summary, the present study provides new insights regarding how perturbation of intracellular ion homeostasis acts as a signal for activation of NLRP3 inflammasome signaling. The data indicate that a rapid decrease in cytosolic [K\(^{+}\)] is a highly efficacious signal for initiating the NLRP3 inflammasome cascade regardless of the presence or absence of coincident increases in cytosolic [Ca\(^{2+}\)]. Despite this dissociation from Ca\(^{2+}\) signaling, the mechanism by which a decrease in [K\(^{+}\)] is coupled to the conformational activation of NLRP3 remains unknown. Moreover, changes in cytosolic [K\(^{+}\)] may also act downstream of NLRP3 at the level of ASC given the suppressive effects of elevated [K\(^{+}\)] on activation of the AIM2/ASC inflammasome in *Francisella*-infected macrophages (60). Perturbation of multiple mitochondrial functions by disruption of the normal cytosolic [K\(^{+}\)]/[Na\(^{+}\)] ratio is a relevant area for investigation (32, 61). However, a recent analysis by Vince and colleagues (62) demonstrated normal NLRP3 inflammasome function in ATP- or nigerin-stimulated murine macrophages that lacked expression of different mitochondrial or mitochondria-associated proteins, including cyclophilin D, Bax, Bak, and parkin, previously implicated in regulation of inflammasome signaling. These findings indicate that linking NLRP3 activation to mitochondrial perturbation by K\(^{+}\) efflux agonists will require consideration of other mitochondrial functions.

**Disclosures**

The authors have no financial conflicts of interest.