Mechanisms of caspase-1 activation by P2X7 receptor-mediated K+ release

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Kahlenberg, J. Michelle, and George R. Dubyak. Mechanisms of caspase-1 activation by P2X7 receptor-mediated K+ release. Am J Physiol Cell Physiol 286: C1100–C1108, 2004.—The mechanisms underlying caspase-1 activation and IL-1β processing during inflammatory activation of monocytes and macrophages are not well defined. Here, we describe an in vitro proteolytic processing assay that allows for comparison of caspase-1 regulatory components in a cell-free system separately from the confounding issue of IL-1β secretion. Analysis of in vitro IL-1β and caspase-1 processing in lysates from unstimulated Bac 1 murine macrophages indicated a slow rate of basal caspase-1 activation and proteolytic maturation of IL-1β. In contrast, brief (5 min) treatment of intact macrophages with extracellular ATP (as an activator of the P2X7 receptor) or nigericin before cell lysis markedly accelerated the in vitro processing of caspase-1 and IL-1β. This acceleration of in vitro processing was strictly dependent on loss of intracellular K+ from the intact cells. The induction of in vitro caspase-1 activation by lysis per se or by K+ loss before lysis was sensitive to pretreatment of intact macrophages with the tyrostatin AG-126 or bromoeanol lactone, an inhibitor of Ca2+-dependent phospholipase A2. Caspase-1 activation and IL-1β processing in lysates from unstimulated macrophages were also accelerated by addition of recombinant ASC, a previously identified adapter protein that directly associates with caspase-1. These data indicate that increased K+ efflux via P2X7 nucleotide receptor stimulation activates AG-126- and bromoeanol lactone-sensitive signaling pathways in murine macrophages that result in stably maintained signals for caspase-1 regulation in cell-free assays.

AG-126; ASC; bromoeanol lactone; IL-1β; inflammation

INTERLEUKIN-1β (IL-1β) is an important proinflammatory cytokine with circulating levels that are tightly regulated to prevent aberrant activation of pathways that can lead to chronic inflammation, septic shock, or death (9). IL-1β accumulates as a 33-kDa procytokine (proIL-1β) in the cytoplasm of monocytes and macrophages, and its activation depends on cleavage to the active, mature 17-kDa form (mIL-1β) by the enzyme caspase-1 (32, 44). Caspase-1 is synthesized as a low-activity 45-kDa zymogen (procaspase-1) that is proteolytically activated by cleavage of its COOH terminus into p10 and p20 subunits. These p10 and p20 subunits assemble to form a tetramer, a homodimer of heterodimers, that is highly active in its ability to cleave and activate proIL-1β (45). In vitro and overexpression studies have suggested that the activation of procaspase-1 depends on the oligomerization of two or more procaspase-1 molecules via caspase association recruitment domain (CARD) interactions between proteins able to bind to the CARD domain of procaspase-1 (12, 31, 35, 36, 40, 46). Martinon et al. (24) recently reported that a four-protein complex, termed the inflammasome, can form in vitro and result in caspase-1 activation. Intermolecular procaspase-1 autocatalytic cleavage is then believed to generate the highly active caspase-1 tetramers (45). However, the intracellular signaling pathways that mediate the activation of caspase-1 in response to IL-1β secretogogues have not been well defined.

Procaspase-1 is constitutively expressed by monocytes and macrophages, its interaction with various regulatory or adapter proteins is presumably controlled by regulated expression of those adapter proteins or acute modulation of the protein-protein interactions. Exposure of monocytes and macrophages to primary inflammatory stimuli, such as LPS/endotoxin, will stimulate proIL-1β production, but little secretion of mIL-1β will occur in the absence of a secondary stimulus that triggers caspase-1 activation (6, 13, 27, 29, 30, 43). Significantly, when LPS-primed monocytes and macrophages are exposed to extracellular ATP, they rapidly release large amounts of processed IL-1β at rates that (depending on cell type) are 5–100 times greater than the amount stimulated by LPS alone. The P2X7 receptor (P2X7-R) mediates this ATP-induced processing/release of IL-1β by a mechanism that requires caspase-1 activation (37, 38). Thus modulation of the intracellular milieu by P2X7-R activation may be the trigger for caspase-1 processing.

The P2X7-R functions as an ATP-gated, nondesensitizing cation channel and an inducer of nonselective macropores permeable to large (≤800 Da) inorganic and organic molecules. Thus activation of this receptor by extracellular ATP rapidly induces a complete collapse of ionic gradients that switches the cytosol from a high K+-low Na+-low Cl−-ionic milieu to a low K+ -high Na+-high Cl− environment (2). Previous studies have demonstrated that a coordinated increase in intracellular Na+ and decrease in intracellular K+ are required at one or more steps in the signaling cascade that couples the P2X7-R to activation of caspase-1 (26, 27, 43). The essential role for K+ release in the activation of caspase-1 is further supported by the robust IL-1β processing induced by stimuli other than ATP that also cause a rapid lowering of cytoplasmic K+ concentration ([K+]i). These include the K+/H+ ionophore nigericin, hypotonicity, staphylococcal α-toxin, and protegrins, antimicrobial peptides released from activated neutrophils (6, 27, 28, 30, 43).

Given the physiological role of caspase-1 in normal inflammatory responses and its pathophysiological roles in chronic degenerative diseases (17, 33), it is important to understand the immediate downstream signaling mechanisms by which K+ release stimuli are coupled to the activation of caspase-1 and the subsequent processing of proIL-1β. However, studies of caspase-1 activation and IL-1β maturation in intact monocyte/macrophages are complicated by the rapid and coincident

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release of IL-1β and caspase-1 to the extracellular space (21, 23, 25, 34, 39). This makes it difficult to dissociate signaling pathways that mediate stimulus-induced caspase-1 activation from the signals required for stimulus-triggered secretion of IL-1β and caspase-1. Here, we describe a novel caspase-1 assay system in which intact, LPS-primed murine macrophages are briefly stimulated with P2X7-R agonists or nigericin immediately before disruption and preparation of cell-free lysates. The cell-free lysates from unstimulated or stimulated cells can then be subjected to comparative analyses of caspase-1 activation and IL-1β processing under defined and readily manipulated in vitro conditions. We report that brief treatment of Bac1 murine macrophages with ATP or nigericin markedly accelerates the rate of in vitro IL-1β and caspase-1 processing. We use this system to show that caspase-1 activation is dependent on K+ loss from the cell and is sensitive to treatment of cells with AG-126 or bromoenol lactone (BEL). Additionally, studies with recombinant murine ASC (mASC) indicate that K+ efflux via P2X7-R activation may function by manipulating protein components known to be involved in caspase-1 activation.

MATERIALS AND METHODS

Reagents and antibodies. Cells were treated with nucleotides (Sigma, St. Louis, MO), nigericin (Calbiochem, San Diego, CA), AG-126 (Calbiochem), BEL (BioMol, Plymouth Meeting, PA), ethacrynic acid (Sigma), and Escherichia coli LPS serotype 0111:04 (List Biologicals, Campbell, CA). mIL-1β ELISA antibodies (PM-425B and MM-425B-B) were obtained from Pierce Endogen (Rockford, IL). Anti-IL-1β used for Western blots (3ZD) was provided by the Biological Resources Branch of the National Cancer Institute-Frederick Cancer Research and Development Center. Other antibodies were obtained as follows: anticaspase-1 p10 rabbit polyclonal antibody for mouse and all HRP-conjugated secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), anti-mASC antibody from Abcam (Science Park, Cambridge, UK), and anti-V5 monoclonal antibody from Invitrogen (Carlsbad, CA).

Plasmids. mASC was cloned from Bac1 macrophages via RT-PCR using the following primers: 5’-GCCATGGGGCGGGCAAGG-3’ and 5’-GCTCTGCTCCAGGTCCATCACCAA-3’. The PCR product was inserted into the pcDNA3.1/V5-His TOPO TA vector according to the manufacturer’s protocol (Invitrogen). The plasmid insert was sequenced using T7 and bovine growth hormone (BGH) reverse primers (Invitrogen).

Transfection. Confluent 10-cm2 plates of COS-1 cells were split 1:3 on the night before transfection. Transfection was done using 2 μg of DNA per plate and Effectene reagent (Qiagen, Valencia, CA) according to the manufacturer’s protocol.

Cell culture. Bac1.2F5 murine macrophages were cultured as previously described (16) in DMEM (containing 44 mM bicarbonate; Sigma) supplemented with 25% L cell conditioned medium, 15% calf serum (HyClone, Logan, UT), and 1% Pen-Strep (100 U/ml penicillin and 100 μg/ml streptomycin; GibCO, Grand Island, NY) in the presence of 10% CO2. For experiments, cells were split 1:3 onto 150-cm2 culture dishes 2–3 days before experiments. COS-1 cells were cultured in DMEM with 10% calf serum and 1% Pen-Strep in the presence of 10% CO2.

IL-1β ELISA. To determine the amount of IL-1β released after ATP stimulation, we used a sandwich ELISA protocol as described previously (13). Briefly, 5 × 105 Bac1 cells were seeded into a 24-well plate. After overnight incubation, the cells were primed with 500 ng/ml LPS for 4 h and washed once with phosphate-buffered saline (PBS), and the medium was replaced with 0.5 ml of a basic salt solution (BSS) consisting of 130 mM NaCl, 5 mM KCl, 20 mM HEPES, pH 7.5, 5 mM glucose, 0.01% BSA, 1.5 mM CaCl2, and 1.0 mM MgCl2. Cells were then stimulated with 1 mM ATP for the indicated time points. The medium was removed and 1–50 μl were added to a BSA-blocked ELISA plate that had been coated overnight with 1 μg/ml anti-murine IL-1β. Biotin-conjugated IL-1β antibody was added, and the plates were incubated at room temperature for 2 h. The plate was washed and incubated with HRP-conjugated streptavidin (Pierce Endogen, Rockford, IL) for 30 min and developed using tetramethylbenzidine as substrate. The absorbance measurements were read with a Molecular Devices SoftMax Pro plate reader and compared with IL-1β standards.

In vitro processing assay. Macrophages (1 × 105) were primed with 500 ng/ml LPS for 4 h. The cells were scraped and washed once in PBS and resuspended in 1 ml of buffer W (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1.0 mM EGTA, and 1.0 mM EDTA) supplemented with 2 mM DTT, 2 μg/ml leupeptin, 100 μg/ml PMSF, and 2.5 μg/ml aprotinin. The cells were then pelleted at 300 g for 30 s, and all but ~50 μl of the buffer was removed. The cells were allowed to swell for 10 min on ice and subsequently lysed by 15 passages through a 22-gauge needle. Lysates were spun at 15,000 g for 15 min, and the supernatant was transferred to a new tube and kept on ice. Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA), and protein levels were adjusted to 22 mg/ml using buffer W. Lysates (10 μl) were aliquoted into 1.5-ml tubes and placed at 30°C to initiate processing. Processing reactions were stopped by addition of an equal volume of 4× SDS-PAGE buffer. Lysates were run on 15% polyacrylamide gels and transferred to PVDF membranes (Millipore, Bedford, NH). Western blots were done with the following antibody concentrations: 5 μg/ml IL-1β, 5 μg/ml caspase-1, and 180 ng/ml V5.

To examine baseline inhibitor effects or ATP- and nigericin-mediated acceleration of IL-1β processing in Bac1 cells, 1 × 106 cells per treatment were primed with 500 ng/ml of LPS for 4 h, washed once with PBS, and then bathed in 10 ml of BSS per plate of cells. Cells were then preincubated for 20 min at 37°C plus or minus inhibitors. Cells were pulsed with ATP or nigericin for 5–30 min, washed, and then lysed as described above.

To examine the role of ASC in vitro processing, COS-1 cells transfected 48 h previously were trypsinized and lysed in buffer W as described above. Transfected or untransfected lysate (1.2 mg) was added to Bac1 cells after they were resuspended in buffer W and most of buffer W was removed, but before they were lysed. After a 10-min incubation on ice, the cells were lysed and incubated at 30°C as described above.

To graphically represent in vitro IL-1β processing, all IL-1β Western blots were scanned and densitometrically measured using Scion Image (www.scioncorp.com). The percentage of processed IL-1β was calculated as follows: pixel density of p17 IL-1β fragment + pixel density of p17 IL-1β fragment + pixel density of p33 IL-1β fragment for each time point.

K+ release assay. Bac1 cells were seeded at 1 × 106 cells per well of a 12-well dish on the night before treatment. Cells were treated with 500 ng/ml LPS for 4 h and washed once with PBS, and the medium was replaced with 1 ml of BSS. The cells were incubated for 20 min with inhibitors and then stimulated with 1 mM ATP for 30 min. The medium was removed, and the cells were lysed in 1 ml of 10% nitric acid. The intracellular K+ content was quantified using atomic absorbance spectroscopy and compared with standards.

RESULTS

Activation of caspase-1 by P2X7-R stimulation at early time points. Study of the proteolytic maturation of caspase-1 and IL-1β in response to ATP and other K+ release stimuli can be confounded by the rapid and near-coincident secretion of these processed proteins from activated cells. LPS-primed Bac1 macrophages stimulated with 1 mM ATP release copious amounts of IL-1β within 30 min as measured by ELISA.
However, during the initial 5 min of ATP stimulation, the release is minimal (Fig. 1A). In contrast, if the cells are pulse stimulated with 1 mM ATP for only 5 min and then transferred to ATP-free medium and incubated for 25 min in the absence of agonist, they release IL-1β in amounts equivalent to that observed in cells continuously stimulated with ATP for the entire 30-min test period (Fig. 1B). Thus, during the initial 5 min of P2X7-R activation, changes occur within the cell that allow for rapid caspase-1 activation and maintenance of that activation, even when the P2X7-R stimulus is withdrawn.

P2X7-R stimulation of intact Bac1 macrophages induces stable signals for regulation of caspase-1 in cell-free lysates. To measure the activation of caspase-1 in response to short pulses of ATP independently of IL-1β secretion, we developed a cell-free assay adapted from previously described methods (24, 45). We prepared highly concentrated, cell-free lysates by disruption of LPS-primed Bac1 cells in hypotonic buffer. These lysates were characterized by a slow, spontaneous activation rate of caspase-1 and coincident IL-1β processing after ~60–120 min of incubation at 30°C, as shown by the accumulation of active p10 caspase-1 fragments and active p17 IL-1β fragments (Fig. 2). Because short pulses of P2X7-R activation are sufficient for a maintained secretion of mature IL-1β, we reasoned that brief exposure of Bac1 macrophages to ATP immediately before hypotonic lysis might initiate the caspase-1 activation process and, consequently, accelerate the processing of caspase-1 and IL-1β during in vitro incubation of the cell-free lysates. To test this, intact Bac1 macrophages were primed with LPS for 4 h and then pulsed with 1 mM ATP for various amounts of time before lysis. ATP pulses as short as 5 min greatly accelerated the in vitro processing of caspase-1 and IL-1β (Fig. 2, B and C), suggesting that ATP induces signaling changes within the intact cells that allow for rapid in vitro processing by the subsequently isolated lysate. ATP activates multiple P2 family receptor subtypes, but most are activated at low (nanomolar to micromolar) concentrations of this nucleotide. The P2X7-R has a lower affinity for ATP, with an EC50 of ~500 μM. If induction of caspase-1 regulatory signals were occurring because of stimulation of P2 family members other than the P2X7-R, lower concentrations of ATP should also accelerate the in vitro processing of IL-1β. However, neither 10 nor 100 μM pulses of ATP for 5 min induced acceleration of IL-1β or caspase-1 processing in vitro, in contrast to the robust activation observed in cells pulsed for 5 min with 1 mM ATP (Fig. 3). This suggests that stimulation of other P2 receptors with higher affinities for ATP is not responsible for the accelerated IL-1β processing phenotype but, rather, is a P2X7-R-specific phenomenon. 3′-O-(4-benzoyl)benzoyl ATP, a more potent P2X7-R agonist, was also able to induce accelerated processing at concentrations as low as 300 μM (data not shown). In contrast, UTP, an agonist for the P2Y2 and P2Y4 receptors expressed in macrophages, was not able to accelerate processing (data not shown). Finally, this ATP-induced acceleration was repressed on preincubation of Bac1 macrophages with oxidized ATP, an antagonist of the P2X7-R before ATP stimulation (data not shown). Taken together, these data indicate that ATP activates the P2X7-R to induce signals or protein complexes necessary for caspase-1 activation and that these signals remain active, even after the cells are lysed. This results in the accelerated processing of IL-1β and caspase-1 in lysates derived from the ATP-stimulated macrophages.

Stimulation of K+ loss from intact cells is necessary for in vitro acceleration of caspase-1 and IL-1β processing. Previous studies have demonstrated that P2X7-R-mediated release of processed IL-1β from intact monocytes or macrophages is dependent on the loss of cytosolic K+ to induce caspase-1 activation. To test whether stimulation of the stable caspase-1-activating components requires loss of K+ from intact cells, we utilized nigericin, a K+/H+ ionophore previously shown to induce processing of IL-1β in human monocytes and murine macrophages (6, 15, 26, 27). LPS-primed Bac1 macrophages were treated with 1 μM nigericin for 5 min before lysis. As with ATP, this nigericin treatment step also accelerated IL-1β and caspase-1 processing in vitro (Fig. 3A). To verify that P2X7-R- and nigericin-dependent acceleration of in vitro caspase-1 activation is dependent on K+ loss from intact macrophages, LPS-primed Bac1 cells were stimulated with 1 mM ATP or 1 μM nigericin for 5 min in test medium containing 130 mM KCl as the major extracellular salt. Stimulation of the intact macrophages in the presence of high extracellular K+ completely blocked the ability of ATP or nigericin to induce accelerated in vitro processing of caspase-1 and IL-1β (Fig. 4, A–C), suggesting that K+ efflux induces intracellular signals that stabilize and enhance the ability of caspase-1 to be subsequently activated in vitro.

![Fig. 1](https://www.ajpcell.org/)

Fig. 1. Bac1 macrophages release IL-1β after 30 min, even with only a 5-min ATP pulse. Bac1 cells were treated with LPS for 4 h, washed, and bathed in basic salt solution (BSS). A: cells were treated with 1 mM ATP for 0, 5, 15, or 30 min, extracellular medium was harvested, and IL-1β release was determined by ELISA. B: cells were treated with 1 mM ATP for 30 min and collected or pulsed with 1 mM ATP for 5 or 15 min, medium was changed and collected after a total time of 30 min, and IL-1β was measured as described in A.
AG-126 and BEL inhibit P2X7-mediated acceleration of in vitro caspase-1 and IL-1β processing. To further investigate how brief P2X7-R activation of intact macrophages induces accelerated caspase-1 and IL-1β processing in cell-free lysates, we tested whether any common signaling components might be required for the basal activation of caspase-1 processing by hypotonic lysis and the acceleration of caspase-1 processing after P2X7-R stimulation. Previous studies have reported that AG-126, a tyrphostin family tyrosine kinase inhibitor, can block inflammatory responses in vivo as well as ATP-mediated caspase-1 activation and secretion of IL-1β and IL-18 from human monocytes (8, 25). The specific target of AG-126 is unknown. In preliminary studies, we verified that AG-126 pretreatment completely represses ATP-induced secretion of mIL-1β from LPS-primed Bac-1 macrophages as measured by ELISA (data not shown). We then incubated Bac-1 cells with 50 μM AG-126 for 20 min before a 5-min pulse with 1 mM ATP followed by lysis and the in vitro processing assay. AG-126

Fig. 2. ATP stimulation induces changes within intact Bac1 macrophages that accelerate caspase-1 activation in vitro. A: schematic representation of methods for ATP-induced acceleration of caspase-1 and IL-1β processing. B: Bac1 cells were treated with LPS for 4 h, washed with PBS, cultured in BSS for 10 min, and then pulsed with 1 mM ATP for 0–30 min. Cells were then lysed and subjected to the in vitro processing assay. Processing was monitored by Western blot for IL-1β and caspase-1 p10 antibodies. C: percentage of IL-1β processing represented graphically using densitometric analysis. Data are from 4 separate experiments.

Fig. 3. ATP stimulates acceleration of processing only when concentrations are sufficient to activate the P2X7-R. Bac1 cells were treated with LPS for 4 h, washed with PBS, cultured in BSS for 10 min, and pulsed with ATP for 5 min. Cells were then lysed, and processing was monitored as described in Fig. 2 legend.
pretreatment of intact macrophages blocked basal IL-1β processing and ATP-stimulated acceleration of IL-1β processing (Fig. 5, A and B). Significantly, direct addition of AG-126 to the in vitro assay after lysis did not block IL-1β processing (data not shown). In vitro caspase-1 activation in lysates from AG-126-treated cells followed the pattern of IL-1β (data not shown). This suggests that the AG-126 target(s) within the intact cell couples P2X7R stimulation to induction of stable signaling pathways or protein complexes that facilitate an increased rate of caspase-1 activation in vitro. Moreover, this AG-126-sensitive pathway also contributes to the "basal" in vitro caspase-1 activation induced by hypotonic lysis.

K⁺ loss from nigerin-treated human monocytes was reported to activate Ca²⁺-independent phospholipase A₂ (iPLA₂), while BEL, an inhibitor of iPLA₂, was able to block nigerin-induced caspase-1 activation (42). Because caspase-1 activation by ATP and nigerin is dependent on K⁺ efflux, we reasoned that the acceleration of in vitro caspase-1 activation observed in lysates from ATP-treated Bac1 cells might also be inhibited by BEL. Preliminary studies indicated that preincubation of Bac1 macrophages with 20 μM BEL strongly inhibited the release of mIL-1β after 30 min of 1 mM ATP stimulation as measured by ELISA (data not shown). We preincubated Bac1 macrophages with 20 μM BEL for 20 min.
and then subjected them to stimulation with 1 mM ATP for 5 min followed by lysis and the in vitro processing assay. Preincubation of intact Bac1 cells with BEL alone blocked the basal rate of caspase-1 and IL-1β processing normally observed in control lysates (Fig. 5B). However, when BEL was added to the cell lysate, it was unable to inhibit in vitro processing of IL-1β or caspase-1 (data not shown). This suggests that hypotonic lysis per se may activate iPLA2 or other BEL-sensitive pathways by decreasing the K+ concentration, thus initiating signals required for in vitro caspase-1 activation. However, once the BEL-sensitive signal is generated, it is not required to sustain the rate of IL-1β processing. Significantly, when BEL-treated Bac1 macrophages were stimulated with ATP before lysis, the accelerated in vitro processing of IL-1β was completely inhibited (Fig. 5A and B). The acceleration of caspase-1 processing was similarly repressed (data not shown). In contrast, preincubation of cells with 150 μM propranolol, which inhibits phosphatidate phosphohydrolase-1 (PAP-1), the other identified target of BEL (10), did not inhibit P2Xγ-mediated acceleration of IL-1β processing (data not shown). This suggests that K+ loss via the P2Xγ receptor activates a BEL-sensitive signaling pathway, most likely iPLA2, that plays a critical role in the generation of caspase-1 regulatory signals that are sustained during cell disruption and subsequent incubation of the cell-free lysates. Finally, neither AG-126 nor BEL inhibits the K+ release triggered by P2XγR activation in intact Bac1 macrophages (Fig. 5C). This indicates that both reagents target caspase-1 regulatory pathways downstream of the K+ release signal itself.

Supplementation of Bac1 lysates with recombinant murine ASC can accelerate in vitro activation of caspase-1. ASC is an adapter protein that has been implicated in caspase-1 activation (3, 35, 36). Because Martinon et al. (24) reported that ASC is an essential component for spontaneous caspase-1 activation in human THP-1 cell-free lysates, we reasoned that ASC might also regulate the activation of caspase-1 in our Bac1 macrophage system. If the role of P2Xγ stimulation of intact cells is to activate stable signals responsible for the activation of caspase-1, then the acceleration of caspase-1 processing observed with P2Xγ stimulation might be mimicked by addition of exogenous ASC to Bac1 lysates. V5-tagged murine ASC (mASC) was expressed in COS-1 cells. Transfected COS-1 cells were then lysed, and 1.2 mg of untransfected or mASC-expressing COS-1 lysate were added to Bac1 macrophages before lysis. The Bac1 cells were then lysed, and in vitro processing was monitored as described for previous experiments. Figure 6 demonstrates that the addition of COS-1 lysate alone did not affect the processing rate of IL-1β; however, addition of COS-1 lysate containing mASC greatly accelerated the rate of IL-1β and caspase-1 processing, mimicking that observed on incubation of intact cells with ATP. Partial immunodepletion of mASC from mASC-expressing COS-1 lysate with a monoclonal antibody to the V5 tag diminished the ability of this lysate to accelerate Bac1 processing (data not shown). Thus, when Bac1 macrophages are disrupted in the presence of exogenous mASC, the lysates rapidly process IL-1β and caspase-1 in vitro, suggesting that rapid in vitro processing may be due to enhanced caspase-1 oligomerization.
and activation. This supports the idea that K⁺ loss from the intact cell by P2X₇ activation may assemble mASC-containing complexes that survive cell lysis and result in rapid in vitro processing of caspase-1 and IL-1β.

**DISCUSSION**

The exact mechanism(s) by which caspase-1 is activated remains unresolved. Primary inflammatory stimuli, such as LPS, upregulate IL-1β synthesis in monocytes and macrophages. However, this LPS treatment elicits only slow rates of caspase-1 activation in most cells, and much of the synthesized IL-1β accumulates as an inactive 33-kDa cytokine within the cytoplasm (9). Acceleration of caspase-1 activation and the resulting IL-1β processing and secretion appear to be dependent on rapid K⁺ release from the monocyte or macrophage. A requirement for such K⁺ loss has been demonstrated for multiple IL-1β secretagogues, including ATP, nigericin, protegrins, and staphyloccocal α-toxin (6, 9, 28, 43). In addition to the role of K⁺ loss, much evidence from studies using heterologous overexpression systems supports the role of CARD-CARD interactions in caspase-1 activation (1, 18, 24, 31, 35). The recent characterization of the so-called inflammasome attributes caspase-1 activation to its oligomerization within the inflammasome complex, in much the same manner in which the apoptosome acts as a platform for activation of apoptotic caspases (24). The inflammasome complex was characterized in human THP-1 monocyte lysates and includes at least four proteins: procaspase-1, ASC/Pycard, NALP1/NAC/DEFCAP/CARD7, and procaspase-5 (24). Caspase-1 activation observed on incubation of THP-1 lysates at 30°C was blocked by incubation with an anti-ASC antibody, suggesting that in vitro activation of caspase-1 is dependent on ASC and inflammasome assembly (24). However, components of the inflammasome have been identified, the signaling mechanisms that link K⁺ flux to assembly of components required for caspase-1 are unknown.

Utilizing an in vitro processing assay, we have been able to elucidate possible signaling mechanisms by which a ligand-gated ion channel is activated to caspase-1 processing. Although a 5-min ATP pulse is too brief to induce measurable release of processed IL-1β from intact Bac1 macrophages [as assayed by ELISA or Western blot (Fig. 1)], it clearly triggers signals or protein complexes of sufficient stability to survive cell disruption and, thereby, results in a much accelerated processing of caspase-1 and IL-1β in vitro. This accelerated rate of in vitro processing is dependent on the induced loss of cytosolic K⁺. Additionally, AG-126 and BEL, two known inhibitors of IL-1β processing and release, blocked the P2X₇-mediated acceleration of caspase-1 activation without affecting K⁺ release. This suggests that these inhibitors act upstream of caspase-1 and that K⁺ loss activates targets of these inhibitors as part of the caspase-1 activation signal. Specifically, the targets of these pharmacological agents may play a role in oligomerization of caspase-1, resulting in its activation. In this regard, we observed that supplementation of Bac1 cell lysates with recombinant mASC, a key scaffolding molecule for inflammasome assembly, can duplicate the acceleration seen with ATP stimulation. These data suggest a mechanism by which activation of P2X₇R channels functions to induce a stable complex, possibly the inflammasome, within the intact macrophage, which provides the platform for efficient caspase-1 activation in vitro.

At least two potential mechanisms may explain how cytosolic K⁺ levels might act to regulate caspase-1 activation. The first is that the basal concentration of cytoplasmic K⁺ could inhibit assembly of complexes needed for caspase-1 oligomerization. CARD-CARD interactions have been shown to be dependent on charge-charge interactions (7), and 130 mM K⁺ in basal cytoplasm may inhibit such binding. When K⁺ release is induced, the local cytosolic [K⁺] may drop sufficiently to allow for CARD-mediated assembly. This is supported by earlier studies showing that caspase-1 activation in THP-1 extracts is repressed by high ionic strength (45).

A second mechanism by which stimulated K⁺ efflux may trigger the caspase-1 cascade is via activation of intracellular signaling pathways. K⁺ efflux has been shown to stimulate Ca²⁺-independent PLA₂ (42), and we have demonstrated that an inhibitor of this enzyme (BEL) prevents ATP-mediated acceleration of caspase-1 activation in Bac1 macrophages. BEL has been shown to also inhibit PAP-1 (10). However, Walev et al. (42) demonstrated that BEL inhibition of nigericin-initiated IL-1β processing was dependent on iPLA₂, rather than PAP-1. This, together with our results showing no inhibitory effect of propranolol, suggests that the BEL-sensitive component important for caspase-1 activation may be iPLA₂. Conceivably, the stimulation of iPLA₂ may be important for generating lipid second messengers that can activate scaffolding molecules involved in caspase-1 activation. Several members of the CATERPILLER family of proteins (14) have been implicated in caspase-1 activation. These proteins, which in-

Fig. 6. Murine ASC (mASC) accelerates caspase-1 and IL-1β processing in Bac1 lysates. COS-1 cells were left untransfected or transfected with mASC. After 2 days, cells were trypsinized and lysed in buffer W. Bac1 cells were treated with LPS for 4 h, and, before lysis, 1.2 mg of COS-1 lysate were added to the Bac1 cells. Cells were lysed, and lysates were subjected to in vitro processing assays at 30°C, and processing was monitored by Western blot using IL-1β and caspase-1 p10 antibodies. Expression of V5-mASC was verified by Western blot using antibodies to the V5 epitope.
clude the inflammasome component NALP-1, contain a nucleotide-binding oligomerization (NACHT) domain and leucine-rich repeats (LRRs). These LRRs are known to be autoinhibitory in some CATERPILLER family members, including some that associate with caspase-1 (41). It is believed that the LRR domains of CATERPILLER family members may be intracellular sensors for regulatory molecules that accumulate in response to infection. Nod1 and Nod2, both members with CARD domains, can become activated in response to peptidoglycan components of bacterial cell walls (4, 5, 11). The putative ligands for the LRR of NALP1 have not been identified, but it has been suggested that “danger” signals may activate NALP1 and, thus, alert the cell to a need for an inflammatory response (4). One potential danger signal could be a lipid second messenger that accumulates during iPLA2 activation. These may bind to, or be recognized by, the LRR of one or more CATERPILLER family members, resulting in conformational changes that induce oligomerization. Recent evidence suggests that saturated fatty acids may be recognized by the LRR of the Toll-like receptor 4 (22). Thus fatty acids released by iPLA2 activity may have a similar function in modulating LRR-containing proteins that participate in caspase-1 activation.

Our data support the second model in which K+ loss activates iPLA2. Preincubation of cells with BEL blocked spontaneous activation of caspase-1 in cell-free Bac1 lysates. This suggests that lysis in the hypotonic, low-[K+] buffer facilitates activation of iPLA2, which then generates a putative lipid second messenger within the lysate for the initiation of caspase-1-activating complexes. If activation of iPLA2 is prevented before or during lysis, no processing of caspase-1 or IL-1β is observed on subsequent incubation of the cell-free lysates at 30°C. P2X7-R activation by ATP appears to activate similar signaling pathways in the intact cell by stimulating increased K+ efflux.

In addition to this possible role of iPLA2, our studies implicate signaling pathways targeted by AG-126, a tyrophostin family tyrosine kinase inhibitor. AG-126 can inhibit ATP-mediated IL-1β processing and release in human monocytes (25). However, the exact target of AG-126 remains unclear. Here, we demonstrate that AG-126 can inhibit the ability of P2X7-R activation to trigger the stable signals that allow for rapid processing of caspase-1 and IL-1β in vitro. Because the activation of several PLA2 isoforms can be influenced by phosphorylation (19), it is conceivable that AG-126 may inhibit a kinase that couples K+ efflux to iPLA2 activation. The molecular target of AG-126 is likely to be important for the inflammatory role of the P2X7-R, because treating mice with AG-126 is able to prevent anti-collagen-induced arthritis, a disease model in which the P2X7-R has also been implicated (8, 20).

Our studies have examined how P2X7-R stimulation results in caspase-1 activation. We have demonstrated the novel finding that stimulation of the P2X7-R is able to induce the acceleration of caspase-1 processing in vitro and that other K+-releasing agents, which act as secondary stimuli for IL-1β processing in macrophages, function similarly. The loss of intracellular K+ induced by P2X7-R activation or the drop in [K+] that accompanies hypotonic lysis appears to be crucial for this response. The ability of both of these mechanisms to induce caspase-1 and IL-1β processing is inhibited by BEL, a known inhibitor of iPLA2, and also by AG-126, a tyrophostin family tyrosine kinase inhibitor. Furthermore, we have shown that addition of mASC to Bac1 lysates mimics the acceleration of processing seen with ATP stimulation, suggesting that components of the inflammasome may be regulated by P2X7-R signaling to facilitate the activation of caspase-1.

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