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Potentiation of Caspase-1 Activation by the P2X7 Receptor Is Dependent on TLR Signals and Requires NF-κB-Driven Protein Synthesis[1]

J. Michelle Kahlenberg,* Kathleen C. Lundberg,† Sylvia B. Kertesy,† Yan Qu,‡ and George R. Dubyak[2†]

The proinflammatory cytokines IL-1β and IL-18 are inactive until cleaved by the enzyme caspase-1. Stimulation of the P2X7 receptor (P2X7R), an ATP-gated ion channel, triggers rapid activation of caspase-1. In this study we demonstrate that pretreatment of primary and Bac1 murine macrophages with TLR agonists is required for caspase-1 activation by P2X7R but it is not required for activation of the receptor itself. Caspase-1 activation by nigericin, a K^+/H^+ ionophore, similarly requires LPS priming. This priming by LPS is dependent on protein synthesis, given that cyclohexamide blocks the ability of LPS to prime macrophages for activation of caspase-1 by the P2X7R. This protein synthesis is likely mediated by NF-κB, as pretreatment of cells with the proteasome inhibitor MG132, or the IκB kinase inhibitor Bay 11-7085 before LPS stimulation blocks the ability of LPS to potentiate the activation of caspase-1 by the P2X7R. Thus, caspase-1 regulation in macrophages requires inflammatory stimuli that signal through the TLRs to up-regulate gene products required for activation of the caspase-1 processing machinery in response to K^+-releasing stimuli such as ATP. *The Journal of Immunology,* **2005, 175: 7611–7622.**

Caspase-1 is the activating enzyme for the proinflammatory cytokines IL-1β and IL-18, which play an important role in inflammatory disease, fever, and septic shock (1). To interact with its receptor, IL-1β must first be cleaved from its 33-kDa pro-form into the 17-kDa active mature cytokine mIL-1β. Caspase-1 is required for this cleavage because caspase-1 knockout macrophages are unable to induce IL-1β cleavage in response to any activating stimuli (2, 3). Given the essential role of caspase-1 in this process, the mechanisms underlying caspase-1 activation have been intensively studied but remain only partially defined. Caspase-1 is constitutively expressed as procaspase-1, a low-activity zymogen that contains an N-terminal caspase recruitment domain (CARD)[3] essential for caspase-1 activation in vivo. Scaffolding proteins with similar CARD, including the adaptor molecule ASC (apoptosis-associated speck-like protein containing a CARD), bind to and oligomerize procaspase-1 within protein complexes termed the inflammasomes, which facilitate caspase-1 autocleavage into p20 and p10 fragments from the C terminus (4–12). These free p20 and p10 fragments assemble into an active heterotetramer that acts as a highly efficient IL-1β converting/cleaving enzyme or ICE (12, 13).

Although constitutively expressed, procaspase-1 remains inactive in the cytoplasm until inflammatory effector cells, such as monocytes and macrophages, receive appropriate stimuli. In these cells, K^+ release stimuli induce rapid and robust activation of caspase-1, resulting in the processing and release of mIL-1β (13–18). One well-characterized physiological K^+ release stimulus is extracellular ATP activation of the P2X7 receptor (P2X7R). Short-term (5 min) stimulation of macrophages with ATP results in the processing and release of IL-1β to the extracellular medium within 15 min (13, 19, 20). However, because IL-1β production is regulated by induced expression as well as proteolysis, such studies necessarily use LPS-primed monocyte/macrophages to up-regulate the transcription and translation of IL-1β before acute stimulation of P2X7R by ATP addition. Recent studies have demonstrated that priming by LPS is also required for efficient caspase-1 activation by ATP-gated P2X7R (4, 5). Thus, even though procaspase-1 and P2X7R are constitutively expressed in non-LPS primed cells, LPS priming is required to facilitate coupling between these two signal transduction proteins. Long-term stimulation of monocytes with LPS in the absence of ATP also induces a slowly developing activation of caspase-1 (16, 17, 21–23); recent data indicate that this action may reflect muramyl dipeptide (MDP) accumulation secondary to intracellular metabolism of the peptidoglycan contaminants in most commercial LPS preparations (24). Regardless of the mechanism, this slow activation by LPS and/or MDP over a time course of several hours contrasts with the rapid (within 5 min) activation of caspase-1 observed with P2X7R stimulation.

LPS signals by binding to a complex of proteins, including serum LPS binding protein, MD-2, CD14, and the extracellular domain of TLR4. TLR4 activation induces recruitment of the adaptor protein MyD88 to the receptor, which results in the formation of a signaling complex that allows for activation of several kinase pathways as well as the nuclear translocation of the transcription factor NF-κB secondary to ubiquitination and degradation of its cytoplasmic inhibitor IκB (25). One recent study has suggested that the adaptor molecules MyD88 and Toll/IL-1R domain-containing

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[3] Abbreviations used in this paper: CARD, caspase recruitment domain; MDP, muramyl dipeptide; mIL-1β, mature IL-1β; BSS, basal salt solution; IKK, IκB kinase; BMDM, bone marrow-derived macrophage; PAMP, pathogen-associated molecular pattern.

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adaptor inducing IFN-β are not required for LPS-mediated priming of ATP-mediated caspase-1 activation (4). However, it is unknown what signaling mechanisms downstream of the TLR facilitate the rapid coupling of ATP-occupied P2X7R to the caspase-1 activation machinery.

Given the complex interplay between LPS priming and P2X7R activation of caspase-1, we have characterized how signal transduction processes induced by short-term LPS priming facilitate the activation of caspase-1 by the P2X7R in both primary mouse bone marrow-derived macrophages (BMDM) and a murine macrophage cell line model. We demonstrate that P2X7R activation of caspase-1 is dependent on prestimulation of macrophages with LPS or other TLR ligands. Moreover, this priming effect is mediated by rapid LPS-induced protein synthesis, likely mediated by the transcription factor NF-κB.

Materials and Methods

Reagents and Abs

Reagents from the following sources were used: lactacystin (Biomol), MG132 (Biomol), Bay 11-7085 (Biomol), *Escherichia coli* LPS serotype O1101:B4 (List Biological Laboratories), monophosphoryl *E. coli* F583 lipid A (Sigma-Aldrich), SB203580 (Biomol), SP600125 (Calbiochem), wortmannin (Sigma-Aldrich), U0126 (Calbiochem), YVAD-cmk (Bachem). Human and murine IL-1β ELISA Abs (M-421B-E, M-420B-B, PM-425B, and MM-425B-B) were from Pierce. Anti-P2X7R was from Alamone Laboratories. 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 (Frederick, MD). Anti-phospho-c-Jun, anti-phospho-Akt, anti-Akt, anti-phospho-ERK1/2, and anti-ERK1/2 Abs were from Cell Signaling. Other Abs were obtained from Santa Cruz Biotechnology anti-mouse caspase-1 p10 rabbit polyclonal, anti-IκBα, anti-actin, and all HRP-conjugated secondaries. The 19-kDa *Mycobacterium tuberculosis* lipoprotein was a gift of Dr. C. V. Harding, III (Case Western Reserve University, Cleveland, OH). CpG DNA 1886 was a gift from Dr. F. Heinzel (Case Western Reserve University, Cleveland, OH).

Cell culture

Bac1.2F5 (Bac1) murine macrophages were cultured as previously described (26) in DMEM supplemented with 25% L cell-cultured medium, 15% calf serum, and 1% penicillin-streptomycin in the presence of 10% CO_2. Cells were split 1:3 onto culture dishes 2–3 days before experiments. Mouse BMDM were obtained as previously described (27). Briefly, femurs from BALB/c mice (6–10 wk) were isolated and the marrow was flushed with 10 ml of DMEM. The cells were washed once with DMEM and then plated and cultured for 9 days in DMEM (Sigma-Aldrich) supplemented with 25% L cell-cultured medium, 15% calf serum (HyClone Laboratories), and 1% penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin; Invitrogen Life Technologies) in the presence of 10% CO_2. The resulting macrophages were detached with PBS supplemented with 5 mM EDTA and 4 mg/ml lidocaine and replated in six-well or 24-well dishes for subsequent experiments. All experiments involving the use of mice were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University. COS-1 cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% calf serum (HyClone Laboratories) and 1% penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin; Invitrogen Life Technologies) in the presence of 10% CO_2.

In vitro assay for processing of caspase-1 and IL-1β

This assay was performed as previously described (20). Briefly, 1×10^8 macrophages were treated with 500 ng/ml LPS for 4 h. Following this, the cells were bathed in a basal salt solution (BSS) containing 130 mM NaCl, 5 mM KCl, 20 mM HEPES, 5 mM glucose, 0.01% BSA, 1.5 mM $CaCl_2$, and 1.0 mM $MgCl_2$ for 10 min followed by 1 mM ATP treatment for 5 min where indicated. The cells were washed once in PBS and resuspended in 1 ml buffer W (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1.0 mM EGTA, 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 and all but ~50 μl of the buffer was removed. The cells were then allowed to swell for 10 min on ice and were subsequently lysed by 15 passages through a 22-gauge needle. Lysates were then centrifuged at 15,000 × *g* for 15 min and the supernatant was removed into a new tube and kept on ice. Protein concentrations were determined using the Bradford assay (Bio-Rad) and protein levels were adjusted to 22 mg/ml using buffer W. A total of 10 μl of lysates were aliquoted into 1.5-ml tubes and were placed at 30°C for the indicated times. Processing reactions were stopped by adding an equal volume of 4× SDS-PAGE buffer. Lysates were run on 15% polyacrylamide gels and transferred to polyvinylidene difluoride (Millipore). Western blots were done with the following Ab concentrations: IL-1β 5 μg/ml, caspase-1 5 μg/ml. To determine the effects of inhibitors on LPS potentiation of P2X7R activation of caspase-1, primary or Bac1 macrophages were preincubated with the inhibitors for 30 min followed by (without removal of the inhibitor) a 3.5–4 h stimulation with 0.5–1 μg/ml LPS. Cells were then washed and treated as described.

K^+ release assay

Bac1 macrophages were seeded 1×10^6 cells per well of a 12-well dish for 12–18 h before experiments. Where indicated, cells were preincubated with various inhibitors for 30 min followed by treatment with 1 μg/ml LPS for a total incubation time of 4 h, following removal of the LPS priming medium. The cells were washed once with PBS, bathed with 1 ml of BSS, and then stimulated with 1 mM ATP for 2–30 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. In experiments in which no inhibitor was used, the cells were treated with or without 1 μg/ml LPS for 4 h and processed as described.

In vitro JNK assay

This reaction was conducted as previously described (26). Briefly, Bac1 macrophages were seeded at 2×10^6 per well. The cells were then treated as previously indicated and the cells were lysed in a 0.1% Triton X-100 buffer (25 mM HEPES (pH 7.5), 300 mM NaCl, 1.5 mM $MgCl_2$, 200 μM EDTA, 0.1% Triton X-100, 500 μM DTT, 20 mM β-glycerophosphate, 2 μg/ml leupeptin, 2.5 μg/ml aprotinin). Lysates were then precipitated using 3 μg of GST-*jun* agarose beads for 4 h at 4°C. Beads were then washed twice in lysis buffer and once in 50 mM HEPES/1 mM DTT. Beads were then incubated with 20 μl of kinase buffer (25 mM Tris (pH 7.4), 0.5 mM DTT, 10 mM $MgCl_2$, 7.5 μM ATP) for 30 min at room temperature. The reactions were stopped with 20 μl of 2× SDS-PAGE sample buffer. The phosphorylation of c-*jun* was monitored by Western blot using an Ab specific for the phosphorylated form of c-*jun*.

Activation of IκB kinase (IKK)

Activation of IKK by LPS was assayed by measuring the rapid degradation of IκB-α. Macrophages on six-well plates were preincubated with various concentrations of MG132 or Bay 11-7045 for 30 min before stimulation with LPS for 5–60 min. The cells were then lysed and processed for SDS-PAGE and Western blotting as described for the caspase-1 activation experiments. The transferred lysates were serially probed with anti-IκB-α and then anti-actin.

Induction of caspase-1 activation and release

To determine the activation of caspase-1 within intact cells, Bac1 cells or primary BMDM were stimulated with 0.5 or 1 μg/ml LPS for up to 4 h. Cells were then washed 1× with PBS and bathed in a sodium gluconate balanced salt solution (130 mM sodium gluconate, 5 mM KCl, 20 mM HEPES, 5 mM glucose, 0.01% BSA, 1.5 mM $CaCl_2$, and 1.0 mM $MgCl_2$) for 10 min at 37°C. This process was followed by stimulation with 1 mM ATP for 30 min. The extracellular medium was collected and the protein was precipitated using TCA and redissolved in SDS-PAGE buffer. The cells were lysed in SDS-PAGE buffer. Both samples were then resolved on a 15% acrylamide SDS-PAGE gel and processed for Western blot analysis of IL-1β and caspase-1 levels.

IL-1β ELISA

To determine the amount of IL-1β released after ATP stimulation, we used a sandwich ELISA protocol as previously described (17). Briefly, 1×10^6 Bac1 macrophages were seeded into a six-well plate. After overnight incubation, the cells were primed with 500 ng/ml LPS or 500 ng/ml lipid A for 4 h, washed once with PBS, and the medium was replaced with 1.0 ml of BSS. Cells were then stimulated with 1 mM ATP for 30 min. The medium was then removed and 1–50 μl was added to a BSA-blocked ELISA plate that had been coated overnight with 1 μg/ml anti-murine IL-1β. Biotin-conjugated IL-1β Ab was then added and the plates were incubated at room temperature for 2 h. The plate was then washed and incubated with HRP-conjugated streptavidin (Pierce) for 30 min and developed using tetramethyl benzidine as substrate. The absorbance measurements were read with a Molecular Devices SoftMax Pro plate reader and were compared with IL-1β standards.

Cleavage of recombinant pro-IL-1β as a measure of caspase-1 activity in cell-free lysates

Confluent 10-cm plates of COS-1 cells were split 1:3 the night before transfection. Transfection was done using 2 μg of human pro-IL-1β DNA (a gift from Dr. S. Mizel, Wake Forest University Baptist Medical Center, Winston-Salem, NC) per plate and Effectene reagent from Qiagen according to manufacturer's protocol. COS-1 cells were then lysed in hypotonic buffer W by shear force with a 22-gauge needle and the protein concentration was adjusted to 15 mg/ml. Bac1 macrophages were treated and prepared as described for the in vitro processing assay, but 18.5 μg of COS-1 lysate protein containing the overexpressed IL-1β was added to 990 μg (45 μl per tube) of Bac1 lysate protein and then the mixed lysate was aliquoted (6 μl per tube) and placed at 30°C for the indicated times. As a control, untransfected COS-1 lysate was also added to Bac1 lysates. To stop processing, the reaction was diluted 1/5 with buffer W and placed on ice. An ELISA specific for the human mIL-1β 17-kDa fragment was used to measure the processed IL-1β as previously described.

Results

Priming by TLR4 is required for caspase-1 activation by the P2X7R

When intact monocytes or macrophages are stimulated by ATP to process and assemble caspase-1 p10/p20 heterotetramers, these active caspase-1 complexes are rapidly released to the extracellular environment, making it difficult to detect active caspase-1 in cell lysates (28). Fig. 1*A* illustrates this rapid P2X7R-dependent release of active caspase-1 in the Bac1 murine macrophage model cell line. When Bac1 macrophages primed with LPS for 4 h were stimulated with 1 mM ATP for up to 30 min, only minor intracellular accumulation of the caspase-1 p10 subunit was observed. This observation contrasted with the marked accumulation of caspase-1 p10 in the extracellular medium after a 5 min lag period following ATP addition. Because recent reports have suggested that LPS and other TLR ligands are required for efficient activation of caspase-1 by ATP stimulation of the P2X7R in peritoneal macrophages (4, 5), we also tested the ability of ATP to induce rapid release of active caspase-1 from parallel samples of Bac1 cells that were not LPS primed. In the absence of LPS priming, minimal active caspase-1 p10 fragment was released into the extracellular medium even after 30 min of ATP stimulation. Significantly, LPS priming did not alter the intracellular level of procaspase-1 (Fig. 1*A*) but did induce significant accumulation of the pro-IL-1β substrate of caspase-1. Fig. 1*B* illustrates that LPS priming was similarly required to facilitate the coupling of P2X7R to caspase-1 activation and release in primary BMDM from BALB/c mice. Moreover, near-maximal activation of caspase-1 by ATP-occupied P2X7R was observed in macrophages primed with LPS for only 60 min; this rapid induction of the coupling machinery preceded the significant accumulation of intracellular pro-IL-1β.

The activation of P2X7R induces rapid K^+ efflux from monocyte/macrophages and previous studies have demonstrated that this K^+ loss is required for caspase-1 activation by ATP-occupied P2X7 (13, 15, 20). Fig. 1*C*, *left*, demonstrates that ATP stimulation induced equally effective release of intracellular K^+ from macrophages with or without LPS priming, indicating that LPS priming does not modulate P2X7R activation by ATP per se. Western blot experiments verified similar levels of P2X7R protein in control or LPS-primed macrophages (see Figs. 4*C* and 5*D*). LPS priming also did not affect the kinetics of this ATP-induced K^+ release (Fig. 1*C*, *right*).

Other pathogen-associated molecular patterns (PAMPs) that can contaminate LPS, such as peptidoglycan, or metabolites of such PAMPs, such as MDP, have recently been implicated in the slow activation of caspase-1 induced by long-term stimulation with LPS (24). Lipid A is free of bacterial polysaccharides and is the active moiety of LPS recognized by TLR4. Significantly, priming of Bac1 macrophages with either lipid A or LPS produced equivalent potentiation of ATP-induced caspase-1 activation as indicated by the cleavage and secretion of mIL-1β detected by either ELISA (Fig. 1*D*) or Western blot (Fig. 1*D*, *inset*). This suggests that the priming of P2X7R-induced caspase-1 activation can be elicited by multiple TLR4 ligands and is unlikely to involve polysaccharide contaminants of LPS.

LPS priming is a general requirement for caspase-1 activation by K^+ release stimuli

As indicated in these experiments (Fig. 1), the analysis of caspase-1 activation in intact macrophages is complicated by the near-simultaneous export of the processed caspase-1 to the extracellular compartment. It is unclear from such intact cell studies whether LPS priming is required for the intracellular assembly of the caspase-1 activation complexes in response to K^+ release stimuli or for the secretion of these assembled complexes into the extracellular medium. We recently described a novel method for determining the activation state of caspase-1 using an in vitro processing assay (20) in which intact cells are pretreated with an activating stimulus (i.e., ATP) followed by immediate lysis in a hypotonic buffer and subsequent incubation of the cell-free lysates at 30°C for various times. The kinetics of IL-1β or caspase-1 processing in vitro reflects the activation state of caspase-1 within assembled inflammasome complexes at the time of cell lysis. This permits dissociation of caspase-1 activation signals from the secretion of active caspase-1 fragments, thus allowing for direct analysis of caspase-1 activation by various stimuli. When LPS primed macrophages are stimulated with ATP, the in vitro processing rate of caspase-1 and IL-1β is greatly accelerated, and this phenomenon is strictly dependent on activation of the P2X7R (20). To verify that LPS is required for efficient activation of caspase-1 by P2X7R stimulation in this system, Bac1 macrophages were treated according to the schematic illustrated in Fig. 2*A*. These macrophages were incubated with or without LPS for 4 h followed by brief (5 min) stimulation with 1 mM ATP; this 5 min stimulation corresponds to the lag time between the ATP occupation of P2X7R in intact cells and the initial appearance of activated caspase-1 in the extracellular medium (Fig. 1*B*). The cells were then immediately lysed and caspase-1 processing was monitored in vitro. Fig. 2*B* (*left end*) shows that lysates from control macrophages, lacking both LPS priming and acute ATP stimulation, were characterized by a very low rate of caspase-1 activation. Previous studies from our group (20) and others (6) have indicated that this result reflects basal assembly of inflammasome components by the hypotonic buffer used to lyse the cells. We previously reported that this low basal rate is the same in lysates from macrophages that were pretreated with LPS but not stimulated with ATP (20). In lysates from macrophages primed with LPS and then stimulated with ATP (Fig. 2*B*, *right end*), this processing rate was greatly accelerated, demonstrating that P2X7R activation in the presence of LPS priming markedly accelerates assembly of the caspase-1 activation complexes. In contrast, when macrophages were not primed by LPS before ATP stimulation, no acceleration of caspase-1 activation was observed in the corresponding cell-free lysates (Fig. 2*B*, *middle*). This indicates that LPS priming is required for the rapid intracellular assembly of the caspase-1 activation machinery in response to P2X7R stimulation independently of the export of caspase-1.

The activation of caspase-1 by the P2X7R can be mimicked by treating monocytes or macrophages with other agents, such as ionophores, that induce K^+ release from the cell (13, 16, 20, 29). To determine whether the dependence on LPS priming is specific

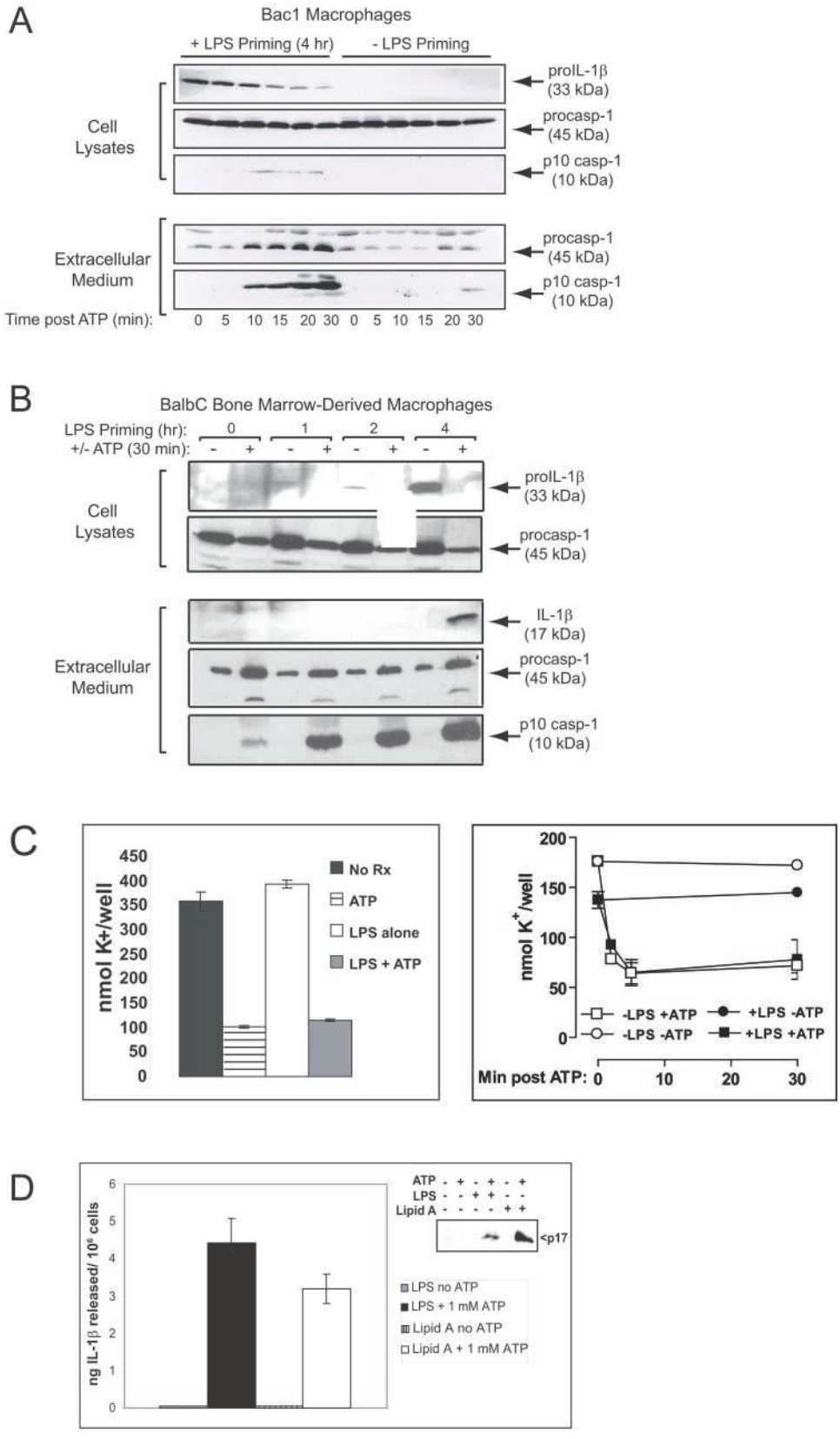

FIGURE 1. Activation of caspase-1 by the P2X7R requires priming with TLR ligands. *A*, Bac1 macrophages were pretreated with or without LPS (500 ng/ml) for 4 h before stimulation with 1 mM ATP for varying times. Extracellular medium samples were collected, TCA precipitated, and the precipitates dissolved in SDS sample buffer. The cells were lysed directly in SDS sample buffer. Both fractions were analyzed via SDS-PAGE and Western blot using Abs against IL-1β and caspase-1. *B*, BALB/c BMDM were primed with LPS for the indicated times before 1 mM ATP stimulation for 30 min as indicated. Extracellular medium and cell lysates were collected and processed as in *A*. Both fractions were analyzed via SDS-PAGE and Western blot using Abs against IL-1β and caspase-1. *C*, Bac1 macrophages (*left*) or primary bone marrow macrophages (*right*) were treated with 1 mM ATP for 30 min (*left*) or for the indicated times (*right*) in the presence or absence of LPS priming (500 ng/ml for 4 h). The extracellular medium was removed and the cells were lysed in 10% nitric acid. The K^+ remaining in the cells after ATP treatment was then measured using atomic spectroscopy. This result is representative of three separate experiments. *D*, Bac1 macrophages were treated with 500 ng/ml LPS or 500 ng/ml lipid A for 4 h, then transferred to BSS and treated with 1 mM ATP for 30 min. The extracellular medium was collected and extracellular mIL-1β was measured via ELISA. The remaining extracellular medium was precipitated with TCA and extracellular IL-1β was measured (*inset*) via SDS-PAGE and Western blot. These data are indicative of three separate and/or triplicate experiments.

to P2X7R or is a feature common to K^+ release stimuli, cell-free lysates were prepared from Bac1 macrophages that were stimulated with 10 μM nigericin for 5 min with or without prior LPS priming. Fig. 2*C* illustrates that nigericin and ATP stimulation of LPS-primed cells yielded cell-free lysates with similarly rapid rates of in vitro caspase-1 activation; however, in the absence of LPS priming, nigericin treatment was unable to induce this rapid activation of caspase-1. This suggests that LPS priming is a general prerequisite for caspase-1 activation by stimuli that induce K^+ release from inflammatory cells.

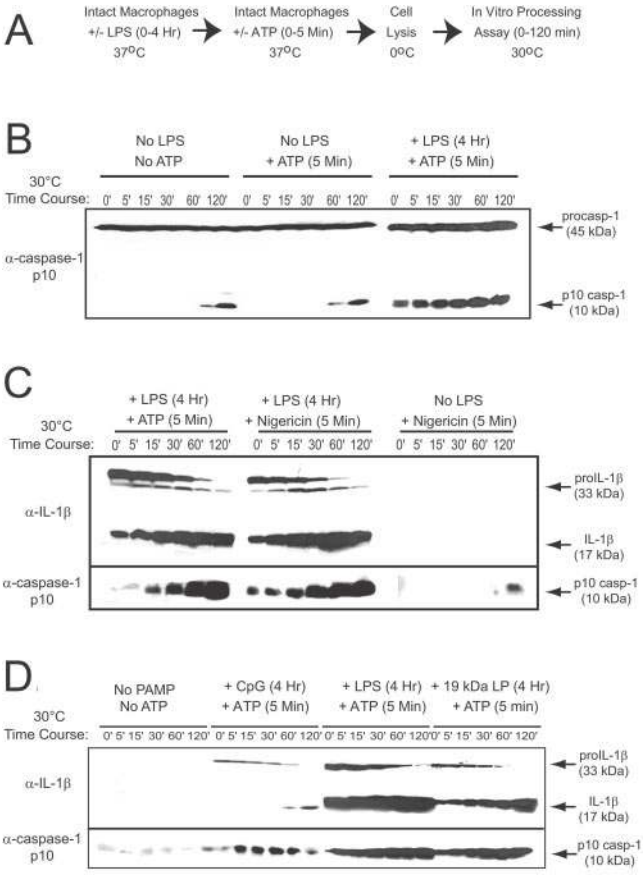

FIGURE 2. Activation of caspase-1 by K^+ loss requires LPS priming. *A*, Schematic of in vitro processing assay. *B*, Bac1 macrophages were pretreated with or without LPS (500 ng/ml) for 4 h and then stimulated with 1 mM ATP for 5 min. The cells were then lysed in hypotonic buffer W and subjected to the in vitro processing assay as described in *Materials and Methods*. The rate of caspase-1 and IL-1β processing was analyzed via SDS-PAGE and Western blot. *C*, Bac1 macrophages were treated with 10 μM nigericin for 5 min in the presence or absence of LPS priming as described in *B*. The cells were then lysed in hypotonic buffer W and subjected to the in vitro processing assay described. *D*, Bac1 macrophages were primed for 4 h with 10 μg/ml unmethylated CpG-DNA 1886, 520 ng/ml *M. tuberculosis* 19-kDa lipoprotein, or 500 ng/ml LPS. The cells were transferred to BSS and then stimulated with 1 mM ATP for 5 min. The cells were immediately lysed in a hypotonic buffer, and the lysates were used in an in vitro processing assay. Processing of IL-1β and caspase-1 were monitored via SDS-PAGE and Western blot for IL-1β and caspase-1 p10 Abs. These blots are representative of at least three separate experiments.

PAMPs that target TLRs other than TLR4 can mimic many responses triggered in macrophages by LPS or lipid A. Because macrophages express TLR2 and TLR9 in addition to TLR4 (30, 31), Bac1 macrophages were primed for 4 h with PAMP-free control media, with 10 μg/ml LPS-free unmethylated CpG DNA 1886 as a TLR9 agonist, with 500 ng/ml LPS as a TLR4 agonist, or with 520 ng/ml 19-kDa lipoprotein from *M. tuberculosis* as a TLR2 agonist. The primed cells were then stimulated with 1 mM ATP for 5 min followed by lysis and the in vitro processing assay as described by Fig. 2*A*. Fig. 2*D* demonstrates that the TLR2 ligand 19-kDa lipoprotein was as efficacious as LPS in priming macrophages for the activation of caspase-1 by the P2X7R. Although CpG DNA also facilitated an increased rate of caspase-1 activation, the response was less robust relative to priming triggered by other the TLR agonists. This likely reflects a global hyporesponsiveness of Bac1 macrophages to CpG DNA given that these cells accumulated less pro-IL-1β relative to that induced by LPS or 19-kDa lipoprotein.

LPS-induced potentiation of caspase-1 activation by ATP requires protein synthesis

A critical downstream consequence of LPS stimulation is the activation of the NF-κB pathway, which results in the up-regulated expression of many proinflammatory gene products (25). Our observation that 60 min of LPS priming was sufficient for near-maximal activation of caspase-1 by P2X7R in intact macrophages (Fig. 1*B*) suggested that putative coupling or regulatory proteins may be characterized be rapid rates of synthesis and/or degradation. Fig. 3*A* demonstrates that a 15 min priming of Bac1 macrophages with LPS followed by ATP stimulation for 5 min was sufficient to trigger an increased rate of caspase-1 processing in the subsequently isolated cell-free lysates. However, maximal induction of the P2X7R-dependent caspase-1 activation was induced by the routine 4 h LPS priming step. This indicates that LPS-mediated priming may involve a rapidly induced but progressive accumulation of proteins required for efficient caspase-1 activation by P2X7R Moreover, pulsing Bac1 cells with LPS for 15 min followed by extensive washing and further incubation for 3.75 h was also sufficient for maximal potentiation of the caspase-1 activation response, suggesting that an early TLR4 signal is sufficient for the priming activity and that maintained TLR4 activation was not required. This would be consistent with the up-regulation of a protein that regulates caspase-1 activation. To determine whether protein synthesis is required for this LPS-mediated priming response, Bac1 macrophages were pretreated with cyclohexamide for 30 min followed by LPS treatment for 4 h. The intact cells were then pulsed with 1 mM ATP for 5 min followed by lysis and the in vitro processing assay. Fig. 3*B* demonstrates that the ability of LPS priming to facilitate caspase-1 activation by ATP-stimulated P2X7R is markedly attenuated in the presence of cyclohexamide. The ability of cyclohexamide to effectively inhibit protein synthesis in these LPS-primed cells was verified by the complete repression of inducible pro-IL-1β accumulation. However, constitutively expressed proteins were not affected by the 4 h cyclohexamide incubation as procaspase-1, and P2X7R levels did not markedly change with this treatment (see Fig. 4*C*). As an additional readout of the relative caspase-1 activities in cell-free lysates from cells primed with LPS in the absence or presence of cyclohexamide, the lysates were supplemented with recombinant human IL-1β as a caspase-1 substrate (Fig. 3*C*). Lysates from LPS primed, ATP-stimulated Bac1 macrophages were characterized by a high rate of recombinant pro-IL-1β cleavage, which was blocked by the caspase-1 inhibitor YVAD-cmk. Lysates from macrophages incubated with cyclohexamide during the LPS priming and ATP stimulation steps were unable to significantly process the recombinant pro-IL-1β.

Cyclohexamide similarly inhibited the ability of LPS to potentiate P2X7R-mediated caspase-1 activation and export in primary BMDM (Fig. 4). Incubation of these cells with this protein synthesis inhibitor during the LPS priming did not alter the intracellular levels of procaspase-1, but completely repressed the ATP-induced accumulation of caspase-1 p10 subunit in the extracellular medium; this was identical with the results observed when the BMDM were stimulated with ATP in the absence of LPS priming (Fig. 4*A*). If the BMDM were first primed with LPS for 3.5 h in the absence of cyclohexamide followed by 30 min of cyclohexamide exposure immediately before the ATP stimulation step, P2X7R-dependent cleavage and secretion of IL-1β could still be observed

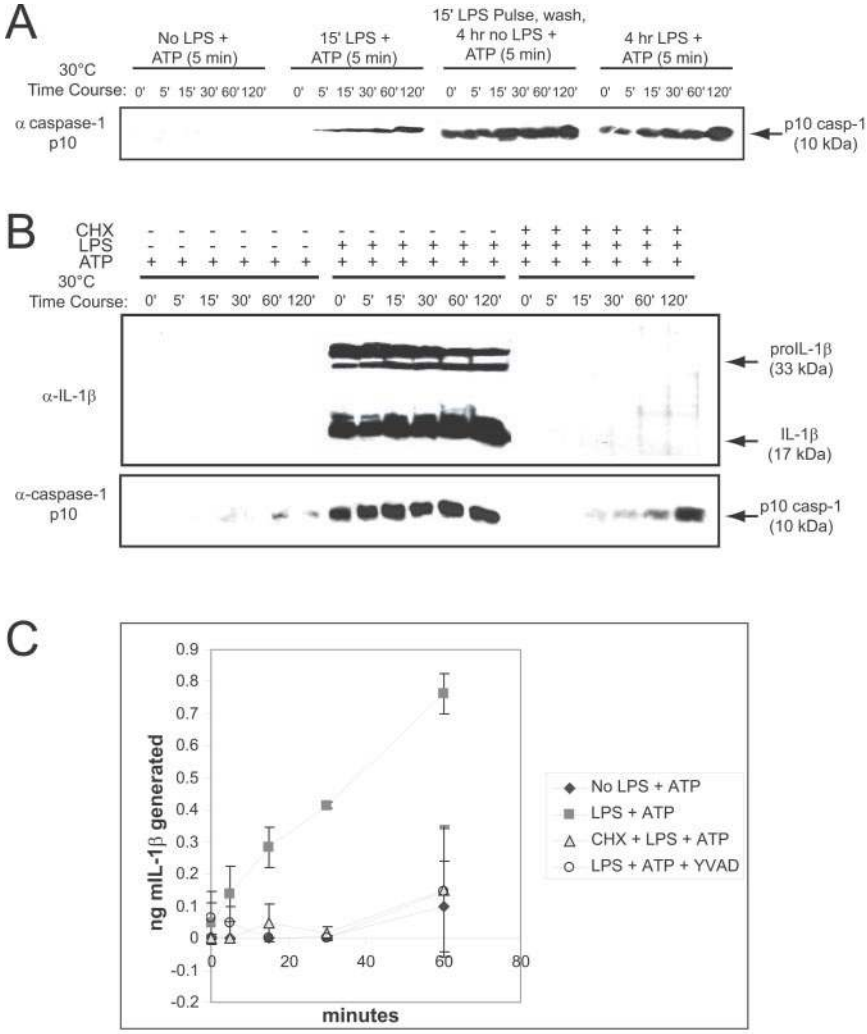

FIGURE 3. LPS priming of caspase-1 activation by P2X7R is dependent on protein synthesis. *A*, Bac1 macrophages were primed with 500 ng/ml LPS for the indicated time points before stimulation with ATP for 5 min. For the 15 min pulse, cells were treated with LPS for 15 min, washed extensively with PBS, and then the medium was replaced for 4 h before ATP treatment for 5 min. Cells were then lysed and caspase-1 activation was monitored by the in vitro processing assay. *B*, Bac1 macrophages were pretreated with 50 μM cyclohexamide for 30 min followed by 4 h of 500 ng/ml LPS where indicated in the presence of cyclohexamide. Cells were then bathed in BSS and stimulated with 1 mM ATP for 5 min followed by lysis and the in vitro processing assay as previously described. The inhibition of protein synthesis by cyclohexamide was determined by Western blot of IL-1β, which is induced by LPS priming. Caspase-1 activation independent of protein synthesis was determined by Western blot for the p10 subunit of caspase-1. *C*, Inhibition of caspase-1 activation by pretreatment with cyclohexamide was confirmed using the cleavage of recombinant human IL-1β as a bioassay. COS-1 cells were transfected with human pro-IL-1β and lysed in hypotonic buffer after 48 h. Bac1 macrophages were primed with LPS or LPS and in the presence of cyclohexamide as indicated. The cells were then treated with 1 mM ATP for 5 min. The cells were then lysed in hypotonic buffer W and 18.75 μg of COS-1 lysate containing recombinant human IL-1β was added to 990 μg Bac1 lysate. The mixture was then aliquoted and incubated at 30°C for the indicated times. The reaction was stopped by dilution 1/5 with buffer W and freezing. The processing of IL-1β was monitored via ELISA specific for human mIL-1β. To ensure that the IL-1β processing reflects caspase-1 activity, lysates were incubated with 10 μM YVAD before transfer to 30°C. All Western blots were repeated at least twice. The bioassay is representative of two experiments done in duplicate.

but at a reduced level (Fig. 4*B*); extracellular accumulation of active caspase-1 was also reduced by this shorter term of exposure to cyclohexamide. However, cyclohexamide pretreatment did not change P2X7R activity as indicated by the K^+ release assay. Bac1 macrophages primed with LPS for 4 h and then treated with 1 mM ATP for 5 min released an average of 90 nmol K/well. In the presence of a 30 min cyclohexamide preincubation followed by an additional 3.5 h of LPS priming with ATP stimulation as described, the macrophages released an average of 84 nmol K/well. This indicates that the 4 h of cyclohexamide exposure did not alter the ability of P2X7R to act as ATP-gated cation channels. Collectively, the various data in Figs. 3 and 4 suggest that a putative coupling protein rapidly accumulates within 15 min of LPS priming and possesses sufficient stability such that acute blockade of ongoing protein synthesis (for 30 min) diminishes, but does not eliminate, the ability of P2X7R to couple to the caspase-1 activation mechanism.

LPS priming of P2X7R-mediated caspase-1 activation requires proteasome activity and NF-κB activation

LPS activation of TLR4 induces MyD88 recruitment to the receptor, followed by IL-1R-associated kinase 1 phosphorylation, and subsequent recruitment and polyubiquitination of TNFR-associated factor 6, which itself can act as an E3 ubiquitin ligase. The proteasome-mediated degradation of several proteins, including IL-1R-associated kinase 1 and IκB, follows these ubiquitination

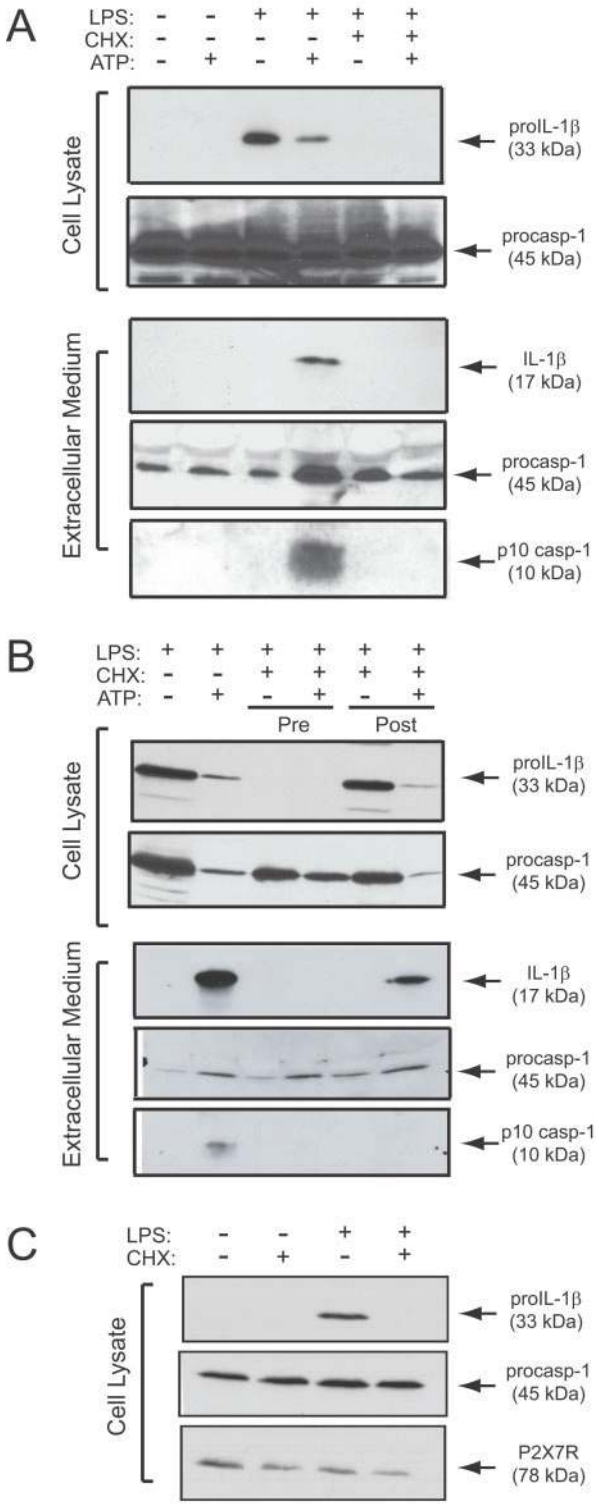

FIGURE 4. LPS priming of caspase-1 activation by P2X7R activation is dependent on protein synthesis in primary macrophages. *A*, BALB/c BMDM were pretreated with or without 50 μM cyclohexamide for 30 min followed by 1 μg/ml LPS for an additional 3.5 h; parallel samples of control cells were neither LPS-primed nor treated with cyclohexamide. Cells were then washed and treated with ATP for 30 min in BSS followed by isolation of the extracellular medium and cell lysates as in Fig. 1*A*. *B*, Cells were treated as in *A* (Pre) or the cells were primed with LPS for 3.5 h followed by a 30 min cyclohexamide addition before washing and stimulation with ATP (Post). The extracellular medium and cell lysates were isolated and processed as in *A*. *C*, Primary BMDM were treated as in *B* without ATP stimulation. Whole cell lysates were analyzed by Western blot for effects of cyclohexamide treatment on IL-1β, procaspase-1, and P2X7R levels.

reactions (32). Because protein synthesis is required for LPS priming of caspase-1 activation by the P2X7R, we tested whether this protein synthesis occurs via proteasome-sensitive mechanisms and NF-κB-mediated transcription. Bac1 macrophages were preincubated with the proteasome inhibitor MG132 for 30 min followed by LPS priming for 3.5 h, pulse stimulation with 1 mM ATP for 5 min before lysis, and analysis by the in vitro processing assay. Preincubation with MG132 was able to block the priming effect of LPS on caspase-1 activation by ATP-stimulated P2X7R (Fig. 5*A*). However, if the macrophages were treated with LPS for 3.5 h followed by a 30 min treatment with MG132, the ability of LPS treatment to potentiate caspase-1 activation by ATP stimulation was not affected. Treatment of the primary BMDM with MG132 before, but not after, LPS priming similarly repressed P2X7R-dependent processing and release of IL-1β (Fig. 5*C*). Preincubation of the cells with MG132 did not affect the ability of activated P2X7R to induce K^+ loss (data not shown), nor did it alter levels of P2X7R protein (Fig. 5*D*). This indicates the effect of MG132 does not reflect a nonspecific action on cell viability or P2X7R expression and function. This suggests that for LPS priming to effectively potentiate caspase-1 activation by the P2X7R, proteasome function must be intact downstream of the TLR signaling complex. To verify that MG132 blocked proteasome activity under the conditions used in the caspase-1 activation experiment, we assayed the cellular levels of IκB in Bac1 macrophages. Pretreatment with MG132 markedly reduced the rapid degradation of IκB triggered by acute LPS stimulation (Fig. 5*B*). Moreover, MG132 completely suppressed the LPS-induced expression of pro-IL-1β (Fig. 5, *A*, *C*, and *D*), a known NF-κB-dependent gene product.

To further address the possible role of NF-κB signaling in LPS priming of the P2X7R→caspase-1 activation cascade, we tested the effects of the IKK inhibitor, Bay 11-7085. Bac1 macrophages were pretreated with increasing concentrations of Bay 11-7085 during the 4 h LPS priming step before the 30 min ATP pulse (Fig. 6*A*). Bay 11-7085 produced a concentration-dependent inhibition of the LPS-dependent expression of pro-IL-1β without affecting the constitutive expression of procaspase-1. At concentrations >1 μM, Bay 11-7085 completely repressed P2X7R-dependent activation of caspase-1 as indicated by the extracellular accumulation of active caspase-1 p10 subunits. A similar inhibitory action of Bay 11-7085 (at 10 μM) was observed in primary BMDM (Fig. 6*B*). Bay 11-7085 did not affect the ability of ATP to induce K^+ release from Bac1 macrophages, indicating that it does not exert direct inhibitory effects on the P2X7R (Fig. 6*C*). Likewise, treatment of primary BMDM with Bay 11-7085 during the 4 h LPS priming incubation did not change the level of P2X7R protein (Fig. 5*D*). Consistent with its action as an IKK inhibitor, Bay 11-7085 also produced a concentration-dependent repression of the rapid IκB-α degradation triggered by LPS (Fig. 6*D*).

LPS potentiation of caspase-1 activation by P2X7R stimulation does not require the ERK, JNK, p38, or PI3K signaling pathways

Additional downstream signaling pathways induced by TLR activation include the ERK, JNK, and p38 families of MAPK as well as PI3K (25, 33). To test whether any of these pathways were involved in the potentiation of caspase-1 activation by the P2X7R, Bac1 macrophages were preincubated with various inhibitors for 30 min followed by LPS treatment for 3.5 h. The cells were then transferred to BSS, treated with 1 mM ATP for 5 min, lysed, and the lysates were incubated at 30°C for the in vitro processing assay. The MEK1 inhibitor U0126, which inhibits the activation of ERK1 and ERK2 by LPS (Fig. 7*B*), did not alter the potentiation

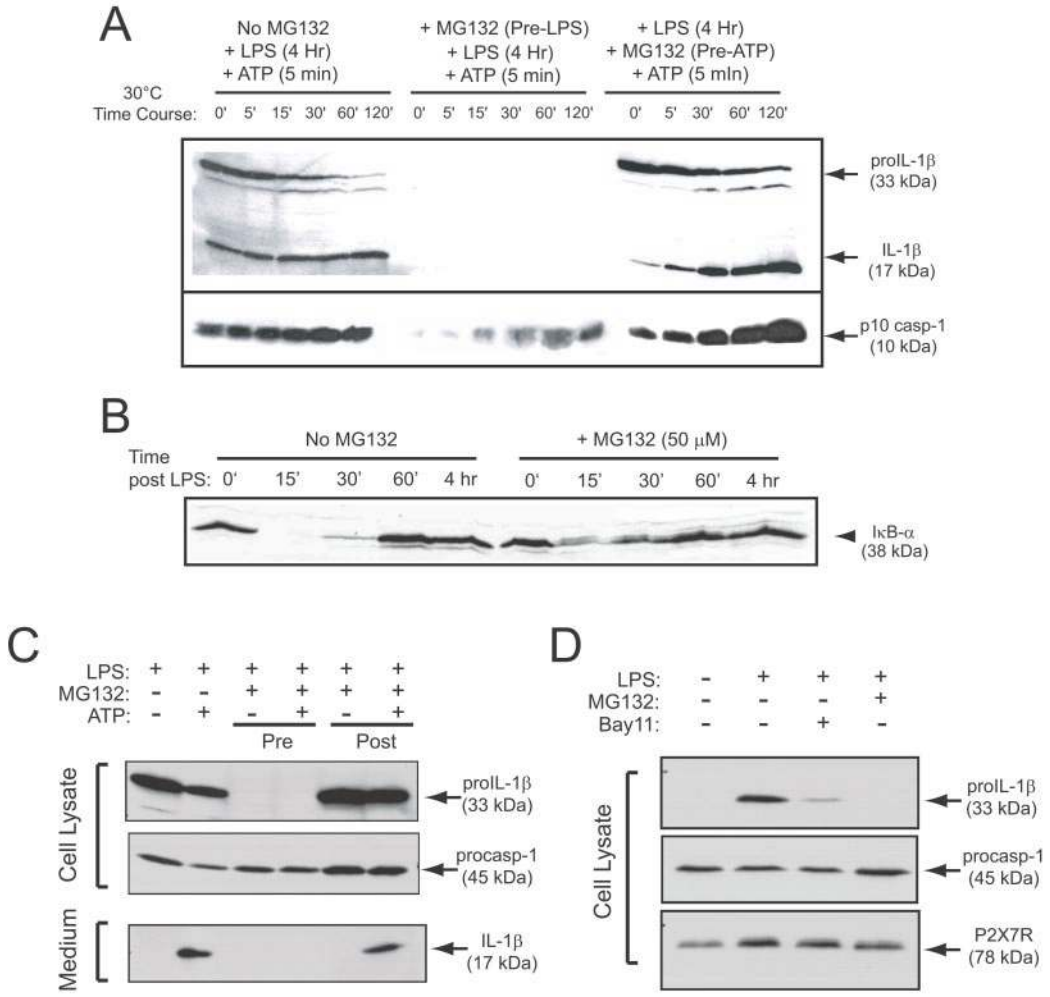

FIGURE 5. LPS priming of caspase-1 activation by the P2X7R requires activity of the proteasome. *A*, Bac1 cells were pretreated with 50 μM MG132 for 30 min followed by priming with LPS for 4 h. The cells were then bathed in BSS, stimulated with 1 mM ATP for 5 min, lysed, and then subjected to the in vitro processing assay. The activation of caspase-1 was monitored by Western blot using Abs for IL-1β and the caspase-1 p10 subunit. This blot is representative of at least three separate experiments. *B*, Bac1 cells were pretreated with 50 μM MG132 for 30 min followed by stimulation with 500 ng/ml LPS for the indicated times before cell lysis. The degradation of IκB was monitored via SDS-PAGE and Western blot. *C*, Primary BMDM were treated with 4 h of LPS alone, 50 μM MG132 for 30 min followed by 3.5 h LPS stimulation (Pre), or LPS for 3.5 h followed by 30 min of 50 μM MG132 (Post). Cells were then washed, bathed in BSS, and stimulated with or without 1 mM ATP for 30 min as indicated. Extracellular medium and cell lysates were processed as in Fig. 1*A* and analyzed by Western blot analysis. *D*, Primary BMDM were pretreated with 50 μM MG132 or 10 μM Bay 11-7085 for 30 min followed by 3.5 h of LPS stimulation. Whole cell lysates were then analyzed for IL-1β, caspase-1, and P2X7R expression by Western blot analysis.

of P2X7R-mediated caspase-1 activation by LPS (Fig. 7*A*). Likewise, SP600125, an inhibitor of JNK signaling, produced no effect on this mode of caspase-1 regulation (Fig. 7*A*) even when tested at concentrations that blocked the activation of JNK by anisomycin in the same cells (Fig. 7*C*). SB203580, a commonly used inhibitor of p38 MAPK, did not impact the activation of caspase-1 by ATP in LPS-primed macrophages but did attenuate the LPS-induced accumulation of IL-1β (Fig. 7, *A* and *D*). Finally, wortmannin, an inhibitor of PI3K, did not reduce the ability of ATP stimulation to activate caspase-1 in LPS primed cells (Fig. 7*E*), even though the same concentration of this inhibitor blocked the phosphorylation of its substrate, Akt, in HEK 293 cells challenged with epidermal growth factor (Fig. 7*F*). These inhibitor studies indicate that the ability of LPS priming to potentiate caspase-1 activation by the P2X7R does not involve an obvious or obligatory role for four of the kinase pathways responsible for many acute responses to LPS and other PAMPs. Additionally, because P2X7R activation has been shown to stimulate each of these four kinase-based signaling pathways in various cell types, (26, 34–37), these data imply that these pathways are not required for direct coupling of ATP-gated P2X7R to the caspase-1 activation machinery.

Discussion

Caspase-1 is the converting enzyme required for the cleavage and maturation of both IL-1β and IL-18. Stimulation of LPS-primed monocytes or macrophages with ATP induces rapid processing and release of these cytokines. Previous studies by Mehta et al. (38) showed that if monocytes are not primed with LPS, ATP treatment cannot induce cleavage of IL-18, which in contrast to IL-1β, is constitutively expressed. This suggested that the efficient coupling of P2X7R to the caspase-1 activation machinery requires one or more signals induced by LPS priming. Two recent reports have described a similar requirement for priming by TLR ligands for ATP-induced activation of caspase-1 in murine peritoneal macrophages (4, 5). In this study, we have characterized the LPS-mediated signaling mechanisms required for this caspase-1 activation by ATP-gated P2X7R. LPS priming appears to be a general

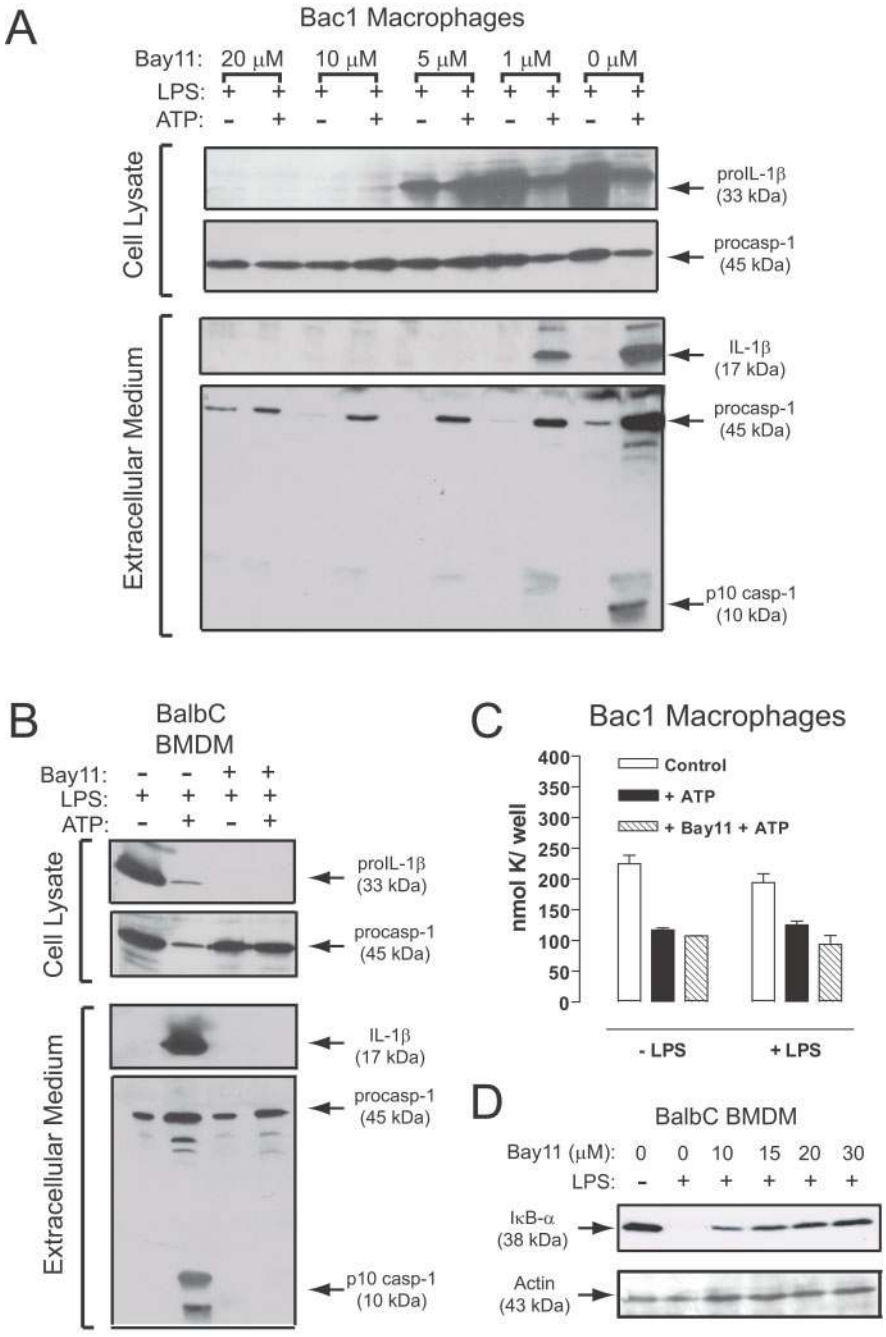

FIGURE 6. The IKK inhibitor, Bay 11-7085, blocks the ability of LPS to potentiate ATP-mediated caspase-1 activation. Bac1 (*A*) or BMDM (*B*) were treated with the indicated concentrations (*A*) or 10 μM (*B*) of Bay 11-7085 for 30 min before priming with 1 μg/ml LPS for 3.5 h. The cells were transferred to BSS and stimulated with 1 mM ATP for 30 min. The extracellular medium and cell lysates were isolated and processed as in Fig. 1*A*. *C*, Bac1 macrophages were treated with 10 μM Bay 11-7085 for 30 min followed by no LPS (*left*) or 1 μg/ml LPS (*right*) for 3.5 h. This process was followed by 30 min of ATP stimulation as indicated. The extracellular medium was removed and the cells were lysed in 10% nitric acid. The K^+ remaining in the cells after ATP treatment was measured using atomic spectroscopy. *D*, BALB/c BMDM were pretreated with the indicated concentrations of Bay 11-7085 for 30 min followed by 30 min of 500 ng/ml LPS stimulation. The cells were lysed in sample buffer and analyzed by SDS-PAGE. The samples were probed using Abs against IκB-α or actin as a loading control.

requirement common to caspase-1 regulation by K^+ release stimuli because activation of caspase-1 by nigericin was similarly dependent on prior treatment of macrophages with LPS. However, LPS pretreatment of macrophages does not affect the ability of ATP to activate the P2X7R, as similar K^+ efflux occurs in response to ATP with or without LPS priming. Because we have previously reported that LPS stimulation of Bac1.2F5 (Bac1) macrophages does not induce ATP release (39), the requirement for LPS priming does not involve autocrine activation of the P2X7R by endogenous ligands. In contrast to our findings, another study, using THP-1 monocytes, suggested that nigericin can induce a cathepsin B cell-dependent caspase-1 activation in the absence of LPS priming (40). We have recently reported that THP-1 monocytes have an increased capacity to accumulate active caspase-1, even in the absence of additional K^+ release stimuli (20, 41). Thus, differences between monocyte vs macrophage model systems may underlie discrepant observations regarding a requirement for LPS priming for robust activation of caspase-1 by various K^+ release stimuli.

We also confirmed that the requisite signals induced by LPS can be mimicked by *E. coli* lipid A, *M. tuberculosis* 19-kDa lipoprotein and to a lesser extent by CpG DNA, suggesting that common signaling pathways downstream of TLR2, TLR4, and TLR9 are involved in facilitating P2X7R-mediated caspase-1 activation. Yamamoto et al. (4) have suggested that the adaptors MyD88 and Toll/IL-1R domain-containing adaptor inducing IFN-β are not required for this TLR-mediated priming effect, but further studies are needed to confirm this observation in several model systems.

Recently, the role of long-term (>24 h) LPS stimulation in the activation of caspase-1 has been questioned. It has been proposed that MDP, derived from the metabolism of peptidoglycan contaminants of LPS, is the key stimulating molecule that induces

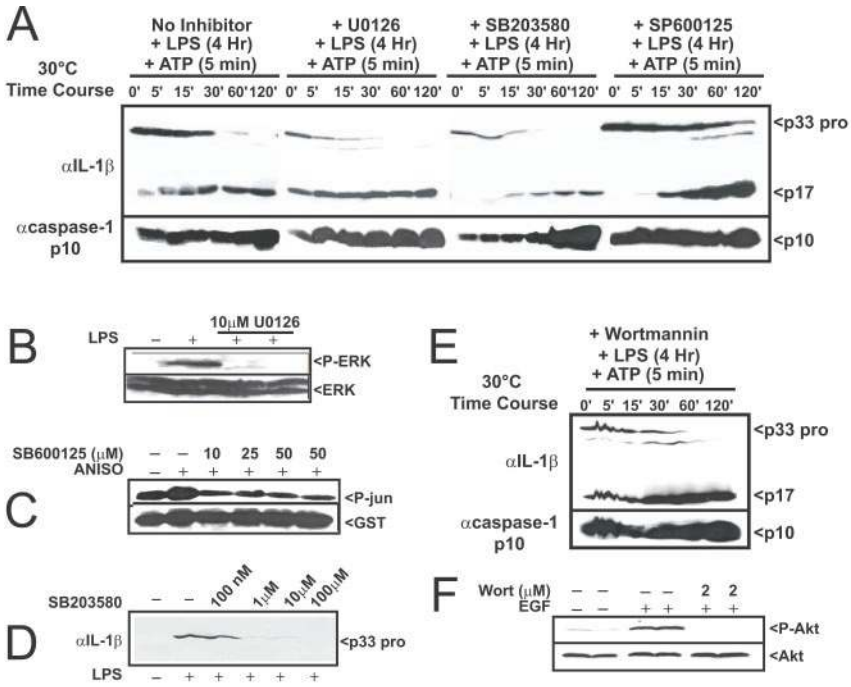

FIGURE 7. LPS priming of caspase-1 activation by the P2X7R does not require the ERK, JNK, p38, or PI3K pathways. *A*, Bac1 macrophages were pretreated with the indicated MAPK inhibitors (10 μM U0126, 1 μM SB203580, or 10 μM SB600125) for 30 min followed by 500 ng/ml LPS stimulation for 4 h in the presence of the inhibitors. The cells were stimulated with 1 mM ATP for 5 min and then lysed in hypotonic buffer. The lysates were used in the in vitro processing assay. IL-1β and caspase-1 processing were monitored by Western blot. *B*, Bac1 macrophages were pretreated with 10 μM U0126 for 30 min and then stimulated with LPS for 10 min as indicated. The inhibition of ERK phosphorylation by U0126 was verified by Western blot for the phosphorylated form of ERK (*top*). Equal amounts of ERK in each lane were also verified by Western blot for ERK (*bottom*). *C*, The ability of SP600125 to inhibit c-*jun* phosphorylation was analyzed by pretreating Bac1 macrophages for 30 min with varying concentrations of SP600125 followed by a 30 min stimulation with 200 ng/ml anisomycin. The cell lysates were then precipitated with GST-*jun* followed by an in vitro kinase assay. The phosphorylation of c-*jun* was analyzed by Western blot using an Ab specific for the phosphorylated form of c-*jun* (*top*). Equal amounts of GST-*jun* substrate per reaction were verified by Western blot using a GST Ab. *D*, Six-well plates of Bac1 macrophages were pretreated with the indicated amounts of SB203580 for 30 min followed by 4 h of stimulation with 500 ng/ml LPS. Cells were lysed in sample buffer and the inhibition of IL-1β up-regulation by SB203580 was monitored by Western blot using an IL-1β Ab. *E*, Bac1 macrophages were pretreated with 2 μM wortmannin for 30 min followed by 500 ng/ml LPS stimulation for 4 h. The cells were stimulated with 1 mM ATP for 5 min, lysed in hypotonic buffer, and used in the in vitro processing assay described for *A*. *F*, HEK293 cells were pretreated as indicated with 2 μM wortmannin for 30 min followed by a 100 ng/ml stimulation with epidermal growth factor for 10 min. Phosphorylation of Akt was measured by SDS-PAGE and Western blot with S^{473} phospho-specific Ab. The membrane was then stripped and reprobed to verify equal levels of Akt. The inhibitor studies are indicative of three separate and/or triplicate experiments.

inflammasome assembly during prolonged LPS exposure (24). MDP, however, does not appear to be a factor in the short-term priming of macrophages for P2X7R-mediated caspase-1 activation because purified lipid A, which is free of peptide moieties, produced the same priming effect as LPS (Fig. 1*D*).

Pretreatment of cells with cyclohexamide before LPS exposure blocked both the up-regulation of IL-1β expression by LPS and the ability of LPS to potentiate the activation of caspase-1 by the P2X7R. Thus, the priming effect of LPS on P2X7R-mediated caspase-1 activation requires protein synthesis. De novo protein synthesis involves LPS-induced gene expression, and our results raised the question as to which transcription factor might be involved. NF-κB is maintained in an inactive state within the cytoplasm by association with IκB. Upon stimulation, IκB is phosphorylated by IKK, ubiquitinated and degraded by the proteasome (42). This allows for translocation of NF-κB to the nucleus and activation of the transcriptional machinery. Preincubation of Bac1 macrophages or primary BMDM with MG132, an inhibitor of the proteasome, or Bay 11-7085, an IKK inhibitor, before LPS treatment blocked the ability of LPS to potentiate caspase-1 activation and to trigger rapid degradation of IκB. In contrast, neither MG132 nor Bay 11-7085 affected P2X7R protein levels (Fig. 5*D*) or P2X7R-dependent K^+ efflux (Fig. 6*C* and data not shown). We also obtained similar results with the proteasome inhibitor lactacystin (data not shown). This requirement for intact proteasome and IKK activity for the LPS priming effects suggests that LPS potentiation of caspase-1 activation by the P2X7R involves activation of NF-κB-dependent gene transcription.

Previous reports have described additional effects of Bay 11 compounds on the ERK, JNK, and p38 MAPK (43). Ligand occupancy of TLRs by their cognate PAMPs activates several well-characterized signaling pathways within monocytes and macrophages. LPS activates all three families of the MAPK, and several reports have implicated PI3K as an important player in mediating downstream effects of TLR signaling (25, 44, 45). However, our pharmacological experiments indicate that these conventional signaling pathways activated by TLR4 are not obligatory for the LPS-dependent priming of caspase-1 activation by the P2X7R (Fig. 7). Thus, the effects of the Bay 11 compounds in our studies are unlikely to involve their effects on any of the MAPK pathways. Additionally, recent reports have identified RIP2/RICK (receptor-interacting protein 2/RIP-like interacting CLARP kinase) as a target for SB203580 (46). Because SB203580 treatment does not affect LPS-mediated potentiation of P2X7R activation of caspase-1, this suggests that RIP2/RICK is most likely not involved in the LPS-mediated potentiation process. This is consistent with studies that used macrophages from RIP2/RICK knockout mice to demonstrate

no requirement for RIP2/RICK in the P2X7R-driven activation of IL-1β processing (47).

What genes are likely targets for up-regulation and facilitation of caspase-1 activation? The oligomerization and activation of caspase-1 is believed to involve assembly of a multiprotein complex, termed the inflammasome. Caspase-1 binds to a scaffold molecule termed ASC/Pycard, which binds to members of the CATERPILLER family that contain both a PYRIN domain and a nucleotide-binding oligomerization (NACHT) domain, including Nalp1, Nalp2, and Nalp3/cryopyrin (6, 8, 9, 11, 48). ASC is the critical molecule within this complex because Abs directed against ASC can disrupt complex formation in vitro (6). In neutrophils and THP-1 monocytes, ASC expression is increased in response to LPS treatment (9, 49). Thus, an increased expression of ASC may overcome interactions with negative regulators such as pyrin, the target of familial Mediterranean fever (48, 50) and several CARD-only proteins such as COP, ICEBERG, and CARD-8 (51–53). This would allow for free ASC to enable efficient assembly of the inflammasome following K^+ release stimuli. In this regard, we have previously reported that addition of recombinant ASC protein to macrophage cell-free lysates induces rapid caspase-1 activation and IL-1β cleavage similar to that induced by P2X7R stimulation of intact macrophages before cell lysis (20).

In addition to ASC, mouse caspase-11 and human caspase-5 are both up-regulated by LPS stimulation (54–56). Both of these proteins have been linked to the activation of caspase-1 and are thought to be functional orthologs (57). Caspase-11 knockout mice are resistant to LPS-induced death and show decreased serum levels of IL-1α and IL-1β after LPS stimulation (55). Additionally, caspase-5 has been identified as a component of one of the inflammasome subtypes (6). A role for NF-κB in caspase-11 up-regulation is supported by reports that identified a binding site for NF-κB on the caspase-11 promoter that becomes occupied in response to LPS (58) and the inhibition of LPS-mediated caspase-11 up-regulation by wedelolactone, an inhibitor of IKK (59). However, other groups have reported that caspase-11 up-regulation is sensitive to pretreatment of cells with the p38 MAPK inhibitor SB203580 (60). This finding would suggest that caspase-11 is not the critical factor required for LPS-mediated potentiation of caspase-1 activation by the P2X7R because SB203580 treatment did not affect caspase-1 activation in response to ATP stimulation (Fig. 7).

Recently, studies have demonstrated that the CATERPILLER protein Nalp3/cyropyrin in human monocytes is also up-regulated by inflammatory stimuli. Significantly, within 30 min of LPS stimulation, a 15-fold increase in Nalp3 mRNA was observed (61). This time frame is consistent with our results showing that a 15 min LPS stimulation was sufficient to prime Bac1 macrophages for P2X7R-dependent caspase-1 activation. In this experiment, the cells were treated for 15 min followed by incubation in BSS for 10 min before lysis, so there was sufficient time for Nalp3 to be up-regulated and allow for caspase-1 activation in response to P2X7R stimulation. Additionally, other reports have demonstrated that the early (0.5–2 h) up-regulation of NF-κB is responsible for detectible IL-1β release in mice injected with LPS (62). Thus, the up-regulation of Nalp3 is an attractive mechanism to explain the role of TLR-mediated priming in P2X7R activation of caspase-1.

The control of caspase-1 activation and subsequent processing of IL-1β and IL-18 is critical for the regulation of inflammatory states in vivo. Understanding how the activation of caspase-1 by endogenous receptors, such as P2X7, can be modulated by bacterial byproducts may contribute to new therapies for the treatment of inflammation and more severe conditions, such as septic shock. These studies show that the activation of caspase-1 by the P2X7R is dependent on prestimulation of the cells with microbially derived ligands for TLR2, TLR4, or TLR9. This prestimulation does not require signaling through known TLR pathway kinases such as ERK, JNK, p38, or PI3K. Additionally, this TLR-mediated potentiation is dependent on protein synthesis and NF-κB function. This indicates that NF-κB-mediated transcription plays an important role in the ability of the P2X7R to activate caspase-1. The specific proteins up-regulated in response to LPS stimulation that allow for efficient caspase-1 activation by P2X7R stimulation may provide an important target for treatment of inflammatory diseases.

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Disclosures

The authors have no financial conflict of interest.

References

1. Dinarello, C. A. 1996. Biologic basis for interleukin-1 in disease. *Blood* 87: 2095–2147.
2. Le Feuvre, R. A., D. Brough, Y. Iwakura, K. Takeda, and N. J. Rothwell. 2002. Priming of macrophages with lipopolysaccharide potentiates P2X7-mediated cell death via a caspase-1-dependent mechanism, independently of cytokine production. *J. Biol. Chem.* 277: 3210–3218.
3. Rowe, S. J., L. Allen, V. C. Ridger, P. G. Hellewell, and M. K. Whyte. 2002. Caspase-1-deficient mice have delayed neutrophil apoptosis and a prolonged inflammatory response to lipopolysaccharide-induced acute lung injury. *J. Immunol.* 169: 6401–6407.
4. Yamamoto, M., K. Yaginuma, H. Tsutsui, J. Sagara, X. Guan, E. Seki, K. Yasuda, S. Akira, K. Nakanishi, T. Noda, and S. Taniguchi. 2004. ASC is essential for LPS-induced activation of procaspase-1 independently of TLR-associated signal adaptor molecules. *Genes Cells* 9: 1055–1067.
5. Mariathasan, S., K. Newton, D. M. Monack, D. Vucic, D. M. French, W. P. Lee, M. Roose-Girma, S. Erickson, and V. M. Dixit. 2004. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430: 213–218.
6. Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-β. *Mol. Cell* 10: 417–426.
7. Poyet, J.-L., S. M. Srinivasula, M. Tnani, M. Razmara, T. Fernandes-Alnemri, and E. S. Alnemri. 2001. Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1. *J. Biol. Chem.* 276: 28309–28313.
8. Srinivasula, S. M., J.-L. Poyet, M. Razmara, P. Datta, Z. Zhang, and E. S. Alnemri. 2002. The PYRIN-CARD protein ASC is an activating adaptor for caspase-1. *J. Biol. Chem.* 277: 21119–21122.
9. Stehlik, C., S. H. Lee, A. Dorfleutner, A. Stassinopoulos, J. Sagara, and J. C. Reed. 2003. Apoptosis-associated speck-like protein containing a caspase recruitment domain is a regulator of procaspase-1 activation. *J. Immunol.* 171: 6154–6163.
10. Grenier, J. M., L. Wang, G. A. Manji, W. J. Huang, A. Al-Garawi, R. Kelly, A. Carlson, S. Merriam, J. M. Lora, M. Briskin, et al. 2002. Functional screening of five PYPAF family members identifies PYPAF5 as a novel regulator of NF-κB and caspase-1. *FEBS Lett.* 530: 73–78.
11. Agostini, L., F. Martinon, K. Burns, M. F. McDermott, P. N. Hawkins, and J. Tschopp. 2004. NALP3 forms an IL-1β-processing inflammasome with increased activity in Muckle-wells autoinflammatory disorder. *Immunity* 20: 319–325.
12. Yamin, T. T., J. M. Ayala, and D. K. Miller. 1996. Activation of the native 45-kDa precursor form of interleukin-1-converting enzyme. *J. Biol. Chem.* 271: 13273–13282.
13. Perregaux, D., and C. A. Gabel. 1994. Interleukin-1β maturation and release in response to ATP and nigericin: evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J. Biol. Chem.* 269: 15195–15203.
14. Perregaux, D. G., R. E. Laliberte, and C. A. Gabel. 1996. Human monocyte interleukin-1β posttranslational processing: evidence of a volume-regulated response. *J. Biol. Chem.* 271: 29830–29838.
15. Perregaux, D. G., and C. A. Gabel. 1998. Human monocyte stimulus-coupled IL-1β posttranslational processing: modulation via monovalent cations. *Am. J. Physiol.* 275: C1538–C1547.
16. Cheneval, D., P. Ramage, T. Kastelic, T. Szelestenyi, H. Niggli, R. Hemmig, M. Bachmann, and A. MacKenzie. 1998. Increased mature interleukin-1β (IL-1β) secretion from THP-1 cells induced by nigericin is a result of activation of p45 IL-1β-converting enzyme processing. *J. Biol. Chem.* 273: 17846–17851.
17. Gudipaty, L., J. Munetz, P. A. Verhoef, and G. R. Dubyak. 2003. Essential role for Ca^{2+} in the regulation of IL-1β secretion by the $P2X_7$ nucleotide receptor in monocytes, macrophages, and HEK293 fibroblasts. *Am. J. Physiol.* 285: C286–C299.

18. Walev, I., K. Reske, M. Palmer, A. Valeva, and S. Bhakdi. 1995. Potassium-inhibited processing of IL-1β in human monocytes. *EMBO J.* 14: 1607–1614.
19. MacKenzie, A., H. L. Wilson, E. Kiss-Toth, S. K. Dower, R. A. North, and A. Surprenant. 2001. Rapid secretion of interleukin-1β by microvesicle shedding. *Immunity* 15: 825–835.
20. Kahlenberg, J. M., and G. R. Dubyak. 2004. Mechanisms of caspase-1 activation by $P2X_7$ receptor-mediated K^+ release. *Am. J. Physiol.* 286: C1100–C1108.
21. Schumann, R. R., C. Belka, D. Reuter, N. Lamping, C. J. Kirschning, J. R. Weber, and D. Pfeil. 1998. Lipopolysaccharide activates caspase-1 (interleukin-1-converting enzyme) in cultured monocytic and endothelial cells. *Blood* 91: 577–584.
22. Grahames, C. B., A. D. Michel, I. P. Chessell, and P. P. Humphrey. 1999. Pharmacological characterization of ATP- and LPS-induced IL-1β release in human monocytes. *Br. J. Pharmacol.* 127: 1915–1921.
23. Perregaux, D. G., P. McNiff, R. Laliberte, M. Conklyn, and C. A. Gabel. 2000. ATP acts as an agonist to promote stimulus-induced secretion of IL-1β and IL-18 in human blood. *J. Immunol.* 165: 4615–4623.
24. Martinon, F., L. Agostini, E. Meylan, and J. Tschopp. 2004. Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome. *Curr. Biol.* 14: 1929–1934.
25. Martin, M. U., and H. Wesche. 2002. Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family. *Biochim. Biophys. Acta* 1592: 265–280.
26. Humphreys, B. D., J. Rice, S. B. Kertesy, and G. R. Dubyak. 2000. Stress-activated protein kinase/JNK activation and apoptotic induction by the macrophage P2X7 nucleotide receptor. *J. Biol. Chem.* 275: 26792–26798.
27. Pai, R. K., M. E. Pennini, A. A. Tobian, D. H. Canaday, W. H. Boom, and C. V. Harding. 2004. Prolonged Toll-like receptor signaling by *Mycobacterium tuberculosis* and its 19-kilodalton lipoprotein inhibits γ interferon-induced regulation of selected genes in macrophages. *Infect. Immun.* 72: 6603–6614.
28. Laliberte, R. E., J. Eggler, and C. A. Gabel. 1999. ATP treatment of human monocytes promotes caspase-1 maturation and externalization. *J. Biol. Chem.* 274: 36944–36951.
29. Perregaux, D., J. Barberia, A. J. Lanzetti, K. F. Geoghegan, T. J. Carty, and C. A. Gabel. 1992. IL-1β maturation: evidence that mature cytokine formation can be induced specifically by nigericin. *J. Immunol.* 149: 1294–1303.
30. Noss, E. H., R. K. Pai, T. J. Sellati, J. D. Radolf, J. Belisle, D. T. Golenbock, W. H. Boom, and C. V. Harding. 2001. Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. *J. Immunol.* 167: 910–918.
31. Gould, M. P., J. A. Greene, V. Bhoj, J. L. DeVecchio, and F. P. Heinzel. 2004. Distinct modulatory effects of LPS and CpG on IL-18-dependent IFN-γ synthesis. *J. Immunol.* 172: 1754–1762.
32. Suzuki, N., S. Suzuki, and W. C. Yeh. 2002. IRAK-4 as the central TIR signaling mediator in innate immunity. *Trends Immunol.* 23: 503–506.
33. Guha, M., and N. Mackman. 2002. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J. Biol. Chem.* 277: 32124–32132.
34. Budagian, V., E. Bulanova, L. Brovko, Z. Orinska, R. Fayad, R. Paus, and S. Bulfone-Paus. 2003. Signaling through P2X7 receptor in human T cells involves $p56^{lck}$, MAP kinases, and transcription factors AP-1 and NF-κB. *J. Biol. Chem.* 278: 1549–1560.
35. Donnelly-Roberts, D. L., M. T. Namovic, C. R. Faltynek, and M. F. Jarvis. 2003. Mitogen-activated protein kinase and caspase signaling pathways are required for $P2X_7$ receptor ($P2X_7R$)-induced pore formation in human THP-1 cells. *J. Pharmacol. Exp. Ther.* 308: 1053–1061.
36. Gendron, F.-P., J. T. Neary, P. M. Theiss, G. Y. Sun, F. A. Gonzalez, and G. A. Weisman. 2003. Mechanisms of $P2X_7$ receptor-mediated ERK1/2 phosphorylation in human astrocytoma cells. *Am. J. Physiol.* 284: C571–C581.
37. Jacques-Silva, M. C., R. Rodnight, G. Lenz, Z. Liao, Q. Kong, M. Tran, Y. Kang, F. A. Gonzalez, G. A. Weisman, and J. T. Neary. 2004. $P2X_7$ receptors stimulate AKT phosphorylation in astrocytes. *Br. J. Pharmacol.* 141: 1106–1117.
38. Mehta, V. B., J. Hart, and M. D. Wewers. 2001. ATP-stimulated release of interleukin (IL)-1β and IL-18 requires priming by lipopolysaccharide and is independent of caspase-1 cleavage. *J. Biol. Chem.* 276: 3820–3826.
39. Beigi, R. D., and G. R. Dubyak. 2000. Endotoxin activation of macrophages does not induce ATP release and autocrine stimulation of P2 nucleotide receptors. *J. Immunol.* 165: 7189–7198.
40. Hentze, H., X. Y. Lin, M. S. Choi, and A. G. Porter. 2003. Critical role for cathepsin B in mediating caspase-1-dependent interleukin-18 maturation and caspase-1-independent necrosis triggered by the microbial toxin nigericin. *Cell Death Differ.* 10: 956–968.
41. Kahlenberg, J. M., and G. R. Dubyak. 2004. Differing caspase-1 activation states in monocyte versus macrophage models of IL-1β processing and release. *J. Leukocyte Biol.* 76: 676–684.
42. Silverman, N., and T. Maniatis. 2001. NF-κB signaling pathways in mammalian and insect innate immunity. *Genes Dev.* 15: 2321–2342.
43. Relić, B., V. Benoit, N. Franchimont, C. Ribbens, M.-J. Kaiser, P. Gillet, M.-P. Merville, V. Bours, and M. G. Malaise. 2004. 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 inhibits Bay 11-7085-induced sustained extracellular signal-regulated kinase phosphorylation and apoptosis in human articular chondrocytes and synovial fibroblasts. *J. Biol. Chem.* 279: 22399–22403.
44. Horák, J. 2003. The role of ubiquitin in down-regulation and intracellular sorting of membrane proteins: insights from yeast. *Biochim. Biophys. Acta* 1614: 139–155.
45. Lee, J. Y., J. Ye, Z. Gao, H. S. Youn, W. H. Lee, L. Zhao, N. Sizemore, and D. H. Hwang. 2003. Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. *J. Biol. Chem.* 278: 37041–37051.
46. Argast, G. M., N. Fausto, and J. S. Campbell. 2005. Inhibition of RIP2/RIck/CARDIAK activity by pyridinyl imidazole inhibitors of p38 MAPK. *Mol. Cell Biochem.* 268: 129–140.
47. Kobayashi, K., N. Inohara, L. D. Hernandez, J. E. Galan, G. Nunez, C. A. Janeway, R. Medzhitov, and R. A. Flavell. 2002. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 416: 194–199.
48. Chae, J. J., H. D. Komarow, J. Cheng, G. Wood, N. Raben, P. P. Liu, and D. L. Kastner. 2003. Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. *Mol. Cell* 11: 591–604.
49. Shiohara, M., S. Taniguchi, J. Masumoto, K. Yasui, K. Koike, A. Komiyama, and J. Sagara. 2002. ASC, which is composed of a PYD and a CARD, is up-regulated by inflammation and apoptosis in human neutrophils. *Biochem. Biophys. Res. Commun.* 293: 1314–1318.
50. Dowds, T. A., J. Masumoto, F. F. Chen, Y. Ogura, N. Inohara, and G. Nunez. 2003. Regulation of cryopyrin/Pypaf1 signaling by pyrin, the familial Mediterranean fever gene product. *Biochem. Biophys. Res. Commun.* 302: 575–580.
51. Humke, E. W., S. K. Shriver, M. A. Starovasnik, W. J. Fairbrother, and V. M. Dixit. 2000. ICEBERG: a novel inhibitor of interleukin-1β generation. *Cell* 103: 99–111.
52. Lee, S. H., C. Stehlik, and J. C. Reed. 2001. COP, a caspase recruitment domain-containing protein and inhibitor of caspase-1 activation processing. *J. Biol. Chem.* 276: 34495–34500.
53. Razmara, M., S. M. Srinivasula, L. Wang, J.-L. Poyet, B. J. Geddes, P. S. DiStefano, J. Bertin, and E. S. Alnemri. 2002. CARD-8 protein, a new CARD family member that regulates caspase-1 activation and apoptosis. *J. Biol. Chem.* 277: 13952–13958.
54. Campos, S. P., Y. Wang, and H. Baumann. 1996. Insulin modulates STAT3 protein activation and gene transcription in hepatic cells. *J. Biol. Chem.* 271: 24418–24424.
55. Wang, S., M. Miura, Y. K. Jung, H. Zhu, E. Li, and J. Yuan. 1998. Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. *Cell* 92: 501–509.
56. Lin, X. Y., M. S. Choi, and A. G. Porter. 2000. Expression analysis of the human caspase-1 subfamily reveals specific regulation of the *CASP5* gene by lipopolysaccharide and interferon-γ. *J. Biol. Chem.* 275: 39920–39926.
57. Van de Craen, M., P. Vandenabeele, W. Declercq, I. Van den Brande, G. Van Loo, F. Molemans, P. Schotte, W. Van Criekinge, R. Beyaert, and W. Fiers. 1997. Characterization of seven murine caspase family members. *FEBS Lett.* 403: 61–69.
58. Schauvliege, R., J. Vanrobaeys, P. Schotte, and R. Beyaert. 2002. Caspase-11 gene expression in response to lipopolysaccharide and interferon-γ requires nuclear factor-κB and signal transducer and activator of transcription (STAT)1. *J. Biol. Chem.* 277: 41624–41630.
59. Kobori, M., Z. Yang, D. Gong, V. Heissmeyer, H. Zhu, Y. K. Jung, M. A. Gakidis, A. Rao, T. Sekine, F. Ikegami, et al. 2004. Wedelolactone suppresses LPS-induced caspase-11 expression by directly inhibiting the IKK complex. *Cell Death Differ.* 11: 123–130.
60. Hur, J., S. Y. Kim, H. Kim, S. Cha, M. S. Lee, and K. Suk. 2001. Induction of caspase-11 by inflammatory stimuli in rat astrocytes: lipopolysaccharide induction through p38 mitogen-activated protein kinase pathway. *FEBS Lett.* 507: 157–162.
61. O'Connor, W., Jr., J. A. Harton, X. Zhu, M. W. Linhoff, and J. P. Ting. 2003. Cutting edge: CIAS1/cryopyrin/PYPAF1/NALP3/CATERPILLER 1.1 is an inducible inflammatory mediator with NF-κB suppressive properties. *J. Immunol.* 171: 6329–6333.
62. Han, S.-J., H.-M. Ko, J.-H. Choi, K. H. Seo, H.-S. Lee, E.-K. Choi, I.-W. Choi, H.-K. Lee, and S.-Y. Im. 2002. Molecular mechanisms for lipopolysaccharide-induced biphasic activation of nuclear factor-κB (NF-κB). *J. Biol. Chem.* 277: 44715–44721.