**Human monocyte stimulus-coupled IL-1β posttranslational processing: modulation via monovalent cations**

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Perregaux, David G., and Christopher A. Gabel. Human monocyte stimulus-coupled IL-1β posttranslational processing: modulation via monovalent cations. *Am. J. Physiol.* 275 (Cell Physiol. 44): C1538–C1547, 1998.—Lipopolysaccharide-activated human monocytes produce pro-IL-1β (pro-IL-1β) but release little of this inflammatory cytokine as the biologically active species. Efficient externalization of mature 17-kDa cytokine requires that the activated monocytes encounter a secondary stimulus such as ATP. To identify cation requirements of the ATP-induced process, lipopolysaccharide-activated monocytes were treated with ATP in media containing different Cl⁻ salts or sucrose. Media devoid of Na⁺ did not support IL-1β processing. Titration of NaCl into choline chloride- or sucrose-based media restored 17-kDa IL-1β production. Na⁺ replacement, however, was not sufficient to support ATP-induced production of 17-kDa IL-1β in the presence of ≥37 mM extracellular K⁺ or Li⁺. Inhibition by K⁺ suggests that efflux of this cation is a necessary component of the stimulus-coupled response. The inhibitory effect achieved by Na⁺ depletion is not due to inactivation of the ATP receptor and is distinct from a caspase-1 inhibitor. Stimulus-coupled IL-1β posttranslational processing, therefore, requires extracellular Na⁺ for a step downstream of the initiating stimulus but preceding caspase-1 activation.

INTERLEUKIN 1 (IL-1) is an important proinflammatory mediator produced in abundance by activated monocytes and macrophages. Two distinct genes encode for IL-1 (α and β) and, despite sharing <30% sequence identity, these two cytokine products signal through shared receptors on target cells and elicit similar biologic responses (8, 43). Human IL-1α and IL-1β are produced as procytokines with apparent molecular masses of 31 kDa (1, 23). Proteolytic activation of pro-IL-1β occurs via caspase-1 (6, 46), the original member of a family of cysteine proteases implicated in apoptotic processes (29, 45), to yield a 17-kDa mature biologically active product; pro-IL-1β is incompetent to bind to IL-1 receptors (25). Caspase-1 also is required for proteolytic activation of pro-IL-18 (13). Importantly, macrophages isolated from mice engineered to lack caspase-1 are impaired in their production of mature IL-1β (20) and IL-18 (13). Moreover, caspase-1-deficient mice are more resistant to lipopolysaccharide (LPS)-induced lethality than their wild-type counterparts (20), and inhibitors of caspase-1 limit inflammatory cytokine production in vitro and in vivo (24, 37, 46). These observations confirm the importance of cytokine posttranslational processing in the generation of active inflammatory mediators. In contrast to pro-IL-1β, pro-IL-1α is fully competent to bind to IL-1 receptors and to elicit cellular signaling pathways (25). Nonetheless, pro-IL-1α may be proteolytically processed to a 17-kDa species, and calpain appears to be responsible for this cleavage (5).

A remarkable feature of pro-IL-1α and pro-IL-1β is their lack of apparent signal peptides, which are required to direct their entry into the secretory apparatus of the cell (23). As a result, the newly synthesized procytokines accumulate within the cytosolic compartment of activated monocytes/macrophages (42). Previous studies indicated that posttranslational processing of pro-IL-1β (defined as proteolytic processing by caspase-1 and release of the mature 17-kDa species to the medium) is an inefficient process in the absence of a secondary stimulus (15, 16, 31, 34). Thus LPS-activated human monocytes produce large quantities of pro-IL-1β, but the vast majority of these newly synthesized polypeptides remains cell associated (19, 34). Release of mature cytokine can be promoted by treating cytokine-producing cells with high concentrations of LPS; this is an inefficient process that appears to work best with freshly isolated monocytes (7, 18, 27). Alternatively, efficient processing can be achieved by treating LPS-activated monocytes/macrophages with extracellular ATP, cytolytic T cells, K⁺-selective ionophores, or bacterial hemolysins (3, 15, 31, 32, 35, 47). This type of stimulus-coupled posttranslational processing is accompanied by cell death, and the dying cells display apoptotic characteristics (14). Although apoptosis traditionally is envisioned as a process whereby cells are eliminated in the absence of an inflammatory response (22), IL-1 release from dying monocytes/macrophages is expected to promote inflammatory processes. Importantly, not all treatments that cause monocyte/macrophage death are sufficient to promote cytokine posttranslational processing (15, 31). The stimulus-coupled mechanism thus is an active, rather than a passive, process.

ATP-induced IL-1β posttranslational processing is mediated via the P₂z (P₂xγ) receptor; this designation is based on the high concentrations of ATP required to activate the cellular response (≥1 mM) and the nature of analogs that can substitute for ATP (9, 19). Ligation of the P₂z receptor leads to major changes in the levels of intracellular ionic components as a result of the opening of porelike channels within the membrane (2, 4, 28, 44). The attendant ionic changes appear to be required for efficient IL-1β posttranslational processing, and treatments that are expected to alter ionic movements impair the cytokine response. For example, replacement of extracellular NaCl with KCl blocks...
ATP-induced cytokine posttranslational processing (32). Likewise, substitution of extracellular Cl− with chaotropic anions (e.g., I− or SCN−) or inclusion of inhibitors of anion transport can block stimulus-coupled IL-1β processing (14, 19, 32, 34). Together, these observations suggest that K+ and anion movements must occur to facilitate the cytokine response. Consistent with this hypothesis is the observation that hypotonic stress, a treatment known to promote K+-Cl− efflux, activates IL-1β posttranslational processing (34, 47). In this study we investigate the ionic requirements for stimulus-coupled processing in more detail. The results indicate that extracellular Na+ is required for efficient ATP-induced IL-1β posttranslational processing and suggest that the cation requirement affects a step distal to P2Z receptor-induced pore opening. These findings further highlight the important role of ionic changes in monocyte/macrophage IL-1 production.

**MATERIALS AND METHODS**

Cells. Human monocytes were isolated from heparinized blood collected from normal volunteers. The mononuclear cell fraction prepared by centrifugation in lymphocyte separation medium (Organon Teknika, Durham, NC) was seeded into six-well cluster dishes (1 × 10^6 cells/well). After 2 h of adherence, nonattached cells were removed and discarded and the adherent monocytes were rinsed twice with RPMI 1640 (RPMI) medium containing 5% fetal bovine serum (FBS; maintenance medium). These cells subsequently were cultured overnight in 2 ml of maintenance medium at 37°C in a 5% CO2 environment.

Metabolic labeling and immunoprecipitation. LPS (serotype 055:B5, Sigma Chemical, St. Louis, MO) was added to the culture medium to achieve a final concentration of 10 ng/ml. Monocytes were activated for 2 h, then the medium was removed and the cells were rinsed once with 2 ml of methionine-free RPMI medium, 20 mM HEPES, pH 7.3, and 1% dialyzed FBS (pulse medium). One milliliter of pulse medium containing 83 µCi/ml of [35S]methionine (Amersham, Arlington Heights, IL; 1,000 Ci/mmol) was added to each well, and the monocytes were labeled for 60 min. The pulse medium subsequently was removed, the wells were rinsed once with an appropriate chase medium, and 1 ml of a chase medium, in the absence or presence of ATP (2 mM) or nigericin (20 µM), was added to initiate posttranslational processing.

After a chase period, media and cell-associated fractions were harvested separately and IL-1β was recovered by immunoprecipitation as previously detailed (34). The resulting immunoprecipitates were analyzed by SDS gel electrophoresis and autoradiography. The quantity of radioactivity associated with media samples and derget lysates of the cell monolayers was determined with a pyruvate detection assay (Sigma Chemical).

86Rb+ efflux assay. Human mononuclear cells isolated as described above were seeded into wells of 24-well cluster plates; on the basis of a cell differential, 2 × 10^6 monocytes were added per well. After 2 h of adherence, nonattached cells were removed, and the adherent monocytes were rinsed with maintenance medium and then incubated overnight in the same medium. Monocytes were loaded with 86Rb+ by replacing the culture medium with 0.5 ml of RPMI medium containing 3% FBS, 20 mM HEPES, pH 7.3, 10 ng/ml LPS, and 6 µCi/ml 86Rb+ (NEN, Boston, MA). After a 3-h incubation with the cation, monocytes were rinsed twice with 0.5 ml of NaCl-based minimal medium containing 1% FBS before the addition of 0.5 ml of fresh minimal medium (with or without Na+) with or without ATP. These cultures were incubated at 37°C for up to 10 min. To harvest the cells, plates were placed on ice and the media samples were collected immediately. Cell monolayers were suspended in 0.5 ml of a lysis buffer composed of 20 mM HEPES, pH 7, 150 mM NaCl, 1% Triton X-100, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM iodoacetic acid; aliquots of the media and cell-derived extracts were analyzed for radioactivity by liquid scintillation counting. The percentage of 86Rb+ recovered extracellularly from each culture subsequently was calculated.

Reagents. Minimal media contained 20 mM HEPES, pH 6.9, 0.9 mM CaCl2, 0.5 mM MgCl2, 2.7 mM KCl, 1.5 mM KH2PO4, 5 mM glucose, 1% FBS, and 137 mM NaCl−, LiCl, choline chloride, or KCl; or 0.25 M sucrose. Media were prepared by dilution of concentrated stock solutions of the individual components (prepared from commercially available solid reagents with the exception of FBS); the pH of each was adjusted to 6.9 with KOH. A microsometer (Precision Systems, Natick, MA) was used to measure osmolarities of 271, 297, 310, 284, and 315 mosM for RPMI medium and the 137 mM NaCl−, LiCl−, KCl−, choline chloride− and sucrose-based minimal media, respectively. Ethacrynic acid, ATP, and nigericin were purchased from Sigma Chemical; a 100 mM stock solution of ATP was prepared and adjusted to pH 7 with KOH before its use. The caspase inhibitor acetyl-tyrosine-valine-alanine-aspartic acid aldehyde (YVAD-CHO) was obtained from Bachem Bioscience (King of Prussia, PA).

**RESULTS**

Na+ depletion from the medium inhibits ATP-induced cytokine release. For this experiment, 17-kDa IL-1β was added to 2 mM ATP in media containing different ionic compositions. When maintained in normal RPMI medium, ATP-treated monocytes exported 17-kDa IL-1β (Fig. 1A, lanes 1 and 2); a smaller amount of 31-kDa pro-IL-1β also was recovered extracellularly. Likewise, monocytes maintained in a minimal 137 mM NaCl− containing medium exported 17-kDa IL-1β in response to ATP (Fig. 1A, lanes 3 and 4). Levels of 17-kDa IL-1β released by cells maintained in RPMI medium or the NaCl-containing minimal medium were comparable, suggesting that special additives to the tissue culture medium (e.g., vitamins and amino acids) were not necessary for the cellular response. However, minimal media in which NaCl was replaced with LiCl, choline chloride, or KCl did not support normal IL-1β posttranslational processing; monocytes maintained in these media released reduced quantities of 17-kDa IL-1β and enhanced levels of the 31-kDa procytokine relative to cultures maintained in an NaCl-based medium (Fig. 1A, lanes 5–10). The magnitude of the increase in pro-IL-1β was not consistent between different experiments; the reason for this is unclear. Monocytes that were treated with ATP in 0.25 M sucrose also failed to release mature cytokine (Fig. 1A, lanes 11 and 12). All minimal media contained, in addition to the 137 mM salt (or 0.25 M sucrose), 20 mM HEPES, pH 6.9, 0.9
mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 2.7 mM KCl, 1.5 mM KH\(_2\)PO\(_4\), 5 mM glucose, and 1% FBS. In all cultures, IL-1\(\beta\) that remained cell associated was recovered primarily as the procytokine species (data not shown).

A previous study reported that IL-1 production by LPS-activated human monocytes required extracellular NaHCO\(_3\); in the absence of NaHCO\(_3\), these cells failed to alkalinize their cytoplasm in response to LPS (30). The NaCl-based minimal medium employed above did not contain added HCO\(_3\)\(^-\), and cells maintained in this medium yielded as much 17-kDa IL-1\(\beta\) in response to ATP as cells maintained in RPMI medium (which contains 24 mM NaHCO\(_3\)). Therefore, the presence of NaHCO\(_3\) did not appear to be required for stimulus-coupled IL-1\(\beta\) posttranslational processing. To ensure that the absence of HCO\(_3\) within the Na\(^+\)-deficient media did not account for the failure of ATP-stimulated monocytes to release 17-kDa IL-1\(\beta\), 5 mM NaHCO\(_3\) was added to the LiCl minimal medium. This addition enhanced extracellular levels of pro-IL-1\(\beta\) but it did not lead to production of the 17-kDa cytokine species (Fig. 1B). A small amount of a 20-kDa polypeptide also was observed in the extracellular immunoprecipitates; identity of this species is unknown.

Membrane transporters that require extracellular Na\(^+\) to function include the Na\(^+\)/H\(^+\) antiporter (12) and the Na\(^+\)-K\(^+\)-ATPase (40). Monocytes treated with ATP in normal RPMI medium in the presence of 5-N,N-diethylamiloride (20 µM), an inhibitor of Na\(^+\)/H\(^+\) antiporters (39), or ouabain (1 mM), an inhibitor of the Na\(^+\)-K\(^+\)-ATPase (40), produced quantities of extracellular IL-1\(\beta\) comparable to those produced by cells maintained in the absence of these inhibitors (data not shown).

Na\(^+\) substitution can restore IL-1\(\beta\) posttranslational processing. Monocytes maintained in the 137 mM choline chloride-based minimal medium did not release significant quantities of radiolabeled IL-1\(\beta\) in the absence of ATP (Fig. 2B, lanes 11 and 12). On the other hand, cells maintained in this medium in the presence of 2 mM ATP exported pro-IL-1\(\beta\) and small quantities of the mature cytokine species (Fig. 2B, lanes 5 and 6; 17-kDa IL-1\(\beta\) produced by these cultures accounted for only 13% of the quantity generated by cells maintained in an NaCl-based medium (Table 1). Cells treated with...
ATP in the choline chloride-based medium released 33% of their total \(^{35}\)S\text{-}methionine-labeled IL-1\(\beta\) in response to ATP (Table 1, experiment A), and those maintained in NaCl- or LiCl-based media released 70% of their overall cytokine product (Table 1). Isotonic media containing combinations of NaCl and choline chloride restored ATP-induced 17-kDa IL-1\(\beta\) production (Fig. 2B, lanes 7–10). Thus, relative to cells maintained in 137 mM NaCl-based medium, monocytes treated with ATP in 45 mM NaCl-92 mM choline chloride or 92 mM NaCl-45 mM choline chloride produced 40 and 79% as much 17-kDa IL-1\(\beta\) (Table 1). Moreover, the proportion of radiolabeled cytokine externalized as Na\(^{+}\) was titrated into the medium (Table 1). Independent of the medium composition, IL-1\(\beta\) that remained cell associated persisted as the procytokine (Fig. 2A).

Monocytes maintained in the 0.25 M sucrose-based medium released minimal quantities of IL-1\(\beta\) in the absence or presence of ATP (Fig. 3, lanes 1–4). As NaCl was titrated into the sucrose-based medium, production of 17-kDa IL-1\(\beta\) in response to ATP again was restored (Fig. 3, lanes 7–10). Monocytes maintained in a medium composed of 90 mM NaCl-83 mM sucrose yielded more 17-kDa IL-1\(\beta\) than did cells maintained in the 137 mM NaCl-based media (Fig. 3, Table 1, experiment B).

In contrast, Na\(^{+}\) replacement did not readily overcome the inhibition evoked by K\(^{+}\) and Li\(^{+}\). Cells maintained in the 137 mM KCl-based medium released large quantities of pro-IL-1\(\beta\) in response to ATP (Fig. 4, lanes 3 and 4), but 17-kDa mature cytokine production was reduced >95% (Table 1, experiment C); cytokine release in the absence of ATP was minimal in the KCl-based medium (Fig. 4, lanes 11 and 12). Replacement of medium K\(^{+}\) with concentrations of Na\(^{+}\) that were sufficient to support IL-1\(\beta\) posttranslational processing in the choline- or sucrose-based media yielded little improvement in production of 17-kDa IL-1\(\beta\) (Fig. 4, lanes 5–10). No 17-kDa species was recovered from monocyte cultures treated with ATP in the presence of 37 mM Na\(^{+}\)-100 mM K\(^{+}\) or 68.5 mM Na\(^{+}\)-68.5 mM K\(^{+}\), respectively. Moreover, when the medium was adjusted to 100 mM Na\(^{+}\)-37 mM K\(^{+}\), the quantity of mature cytokine produced represented only 6% of that generated.

### Table 1. Medium composition alters IL-1\(\beta\) posttranslational processing

<table>
<thead>
<tr>
<th>Base Medium</th>
<th>Total IL-1(\beta), (^{35})S counts</th>
<th>Extracellular IL-1(\beta), % of control</th>
<th>IL-1(\beta) Released, % of total</th>
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<tbody>
<tr>
<td>137 mM NaCl</td>
<td>10,450</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>137 mM LiCl</td>
<td>8,396</td>
<td>5.7</td>
<td>71</td>
</tr>
<tr>
<td>137 mM choline chloride</td>
<td>10,627</td>
<td>13</td>
<td>33</td>
</tr>
<tr>
<td>92 mM NaCl-45 mM choline chloride</td>
<td>10,859</td>
<td>79</td>
<td>67</td>
</tr>
<tr>
<td>45 mM NaCl-92 mM choline chloride</td>
<td>10,307</td>
<td>40</td>
<td>49</td>
</tr>
<tr>
<td>137 mM choline chloride w/o ATP</td>
<td>5,647</td>
<td>ND</td>
<td>ND</td>
</tr>
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Gels containing immunoprecipitated radiolabeled interleukin-1\(\beta\) (IL-1\(\beta\)) were analyzed by a phosphorimager, and radioactivity associated with 31-, 28-, and 17-kDa forms of IL-1\(\beta\) was determined. Total IL-1\(\beta\) counts indicates sum of all species (intracellular and extracellular); quantity of radioactivity associated with 17-kDa species was multiplied by a factor of 2 to correct for loss of radioactivity resulting from caspase-1 cleavage. Quantity of radioactivity associated with extracellular 17-kDa species is indicated as a percentage of control (value observed in cultures treated with ATP in minimal NaCl-based medium). Percentage of IL-1\(\beta\) released was calculated by summing all extracellular species (again by applying 2-fold correction factor for 17-kDa species) and dividing by total quantity of radiolabeled IL-1\(\beta\) recovered from cultures (>100). All cultures were treated with 2 mM ATP in indicated base medium unless designated without (w/o) ATP. ND, no significant radioactivity detected.

ATP in the choline chloride-based medium released 33% of their total \(^{35}\)S\text{-}methionine-labeled IL-1\(\beta\) in response to ATP (Table 1, experiment A), and those maintained in NaCl- or LiCl-based media released 70% of their overall cytokine product (Table 1). Isotonic media containing combinations of NaCl and choline chloride restored ATP-induced 17-kDa IL-1\(\beta\) production (Fig. 2B, lanes 7–10). Thus, relative to cells maintained in 137 mM NaCl-based medium, monocytes treated with ATP in 45 mM NaCl-92 mM choline chloride or 92 mM NaCl-45 mM choline chloride produced 40 and 79% as much 17-kDa IL-1\(\beta\) (Table 1). Moreover, the proportion of radiolabeled cytokine externalized as Na\(^{+}\) was titrated into the medium (Table 1). Independent of the medium composition, IL-1\(\beta\) that remained cell associated persisted as the procytokine (Fig. 2A).

Monocytes maintained in the 0.25 M sucrose-based medium released minimal quantities of IL-1\(\beta\) in the absence or presence of ATP (Fig. 3, lanes 1–4). As NaCl was titrated into the sucrose-based medium, production of 17-kDa IL-1\(\beta\) in response to ATP again was restored (Fig. 3, lanes 7–10). Monocytes maintained in a medium composed of 90 mM NaCl-83 mM sucrose yielded more 17-kDa IL-1\(\beta\) than did cells maintained in the 137 mM NaCl-based media (Fig. 3, Table 1, experiment B).

In contrast, Na\(^{+}\) replacement did not readily overcome the inhibition evoked by K\(^{+}\) and Li\(^{+}\). Cells maintained in the 137 mM KCl-based medium released large quantities of pro-IL-1\(\beta\) in response to ATP (Fig. 4, lanes 3 and 4), but 17-kDa mature cytokine production was reduced >95% (Table 1, experiment C); cytokine release in the absence of ATP was minimal in the KCl-based medium (Fig. 4, lanes 11 and 12). Replacement of medium K\(^{+}\) with concentrations of Na\(^{+}\) that were sufficient to support IL-1\(\beta\) posttranslational processing in the choline- or sucrose-based media yielded little improvement in production of 17-kDa IL-1\(\beta\) (Fig. 4, lanes 5–10). No 17-kDa species was recovered from monocyte cultures treated with ATP in the presence of 37 mM Na\(^{+}\)-100 mM K\(^{+}\) or 68.5 mM Na\(^{+}\)-68.5 mM K\(^{+}\), respectively. Moreover, when the medium was adjusted to 100 mM Na\(^{+}\)-37 mM K\(^{+}\), the quantity of mature cytokine produced represented only 6% of that gener-
ated by cells maintained in the 137 mM NaCl-based medium (Table 1). Addition of Na\(^+\) to the medium, however, did decrease the total amount of radiolabeled IL-1\(\beta\) released in response to ATP (Fig. 4, Table 1).

Likewise, isotonic media containing combinations of LiCl and NaCl did not support ATP-induced IL-1\(\beta\) posttranslational processing. Monocytes maintained in the 137 mM LiCl-based medium released no significant IL-1\(\beta\) in the absence of ATP, but addition of the nucleotide triphosphate stimulated externalization of the procytokine species (Table 1, experiment D). Partial replacement of Li\(^+\) with Na\(^+\) caused less 31-kDa pro-IL-1\(\beta\) to be externalized, but formation of the 17-kDa species remained impaired at all tested Li\(^+\)-Na\(^+\) combinations (Table 1).

Recovery of total [\(^{35}\)S]methionine-labeled IL-1\(\beta\) after the chase period was not constant between different culture conditions within an individual experiment (Table 1). This variance is attributed to differences in the rate of cytokine turnover and/or recovery of immuno precipitable cytokine from an immunologically latent pool (34). Differences in the absolute quantity of radiolabeled IL-1\(\beta\) produced between separate experiments, on the other hand, reflect differences in cell number and/or specific activity of [\(^{35}\)S]methionine employed in the labeling reactions.

Mechanism of pro-IL-1\(\beta\) release from ATP-treated cells maintained in Na\(^+\)-deficient medium is distinct from that employed to release mature IL-1\(\beta\). Kinetics of ATP-induced IL-1\(\beta\) release were compared in NaCl- and LiCl-based minimal media. In the NaCl-based medium, extracellular 17-kDa IL-1\(\beta\) was observed after 30 min of treatment, and the amount of this species increased eightfold by extending the treatment time to 60 min (Fig. 5A). At this time, extracellular radiolabeled IL-1\(\beta\) (sum of the mature and procytokine species and corrected for the 2-fold loss of methionines resulting from caspase-1 cleavage) accounted for >40% of the total (sum of intracellular and extracellular species) radiolabeled IL-1\(\beta\) recovered (Fig. 5B). Relative to the ATP response observed in the presence of NaCl, release of pro-IL-1\(\beta\) from cells maintained in the LiCl-based medium was delayed. After 15 and 30 min of ATP treatment in the LiCl-based medium, <2% of the total radiolabeled IL-1\(\beta\) was externalized, and only 12% was released after 60 min (Fig. 5B). Extracellular levels of IL-1\(\beta\) recovered from the Na\(^+\)-deficient cultures after 120 min of ATP treatment in the absence of Na\(^+\) matched those produced by a 60-min treatment in the presence of the cation (Fig. 5B).

Anion transport inhibitors such as ethacrynic acid previously were shown to prevent formation and release of 17-kDa IL-1\(\beta\) from ATP-treated human monocytes (19). In the NaCl-based medium, 10 µM ethacrynic acid blocked formation of the extracellular 17-kDa
species >90% (Fig. 6, A and B); release of the 31-kDa species, on the other hand, was reduced only 25%. When treated with ATP in the choline chloride-based medium, LPS-activated monocytes released small amounts of 17-kDa IL-1β relative to cells maintained in NaCl-based medium (Fig. 6A). Formation of the mature cytokine species again was inhibited >90% in the presence of ethacrylic acid, but release of pro-IL-1β into the choline chloride medium was largely unaffected (Fig. 6B). Likewise, release of 31-kDa pro-IL-1β by LiCl-maintained monocytes was reduced by only 31% in the presence of ethacrylic acid (Fig. 6, A and B).

The modest inhibition of pro-IL-1β release observed in the presence of ethacrylic acid contrasted sharply with this agent’s effectiveness as an inhibitor of procytokine release from monocytes maintained in the presence of a caspase-1 inhibitor. Relative to monocytes treated with ATP in the absence of an inhibitor, ethacrylic acid and the caspase inhibitor YVAD-CHO (46) blocked mature cytokine formation (Fig. 6C). In contrast to ethacrylic acid-treated monocytes, however, cells treated with the caspase inhibitor externalized elevated quantities of pro-IL-1β (Fig. 6C). Monocytes incubated simultaneously with ethacrylic acid and YVAD-CHO released quantities of pro-IL-1β compa-
rable to those released by the inhibitor-free cultures and no mature cytokine (Fig. 6C).

Na\textsuperscript{+} is required for a step distal to the P\textsubscript{2z} receptor. To explore the possibility that Na\textsuperscript{+} was required for binding of ATP to and/or operation of the P\textsubscript{2z} receptor, LPS-activated, \([^{35}\text{S}]\)methionine-labeled monocytes were stimulated with nigericin to promote release of mature IL-1\textbeta. This K\textsuperscript{+}/H\textsuperscript{+}-exchange ionophore activates IL-1\textbeta posttranslational processing in the absence of ATP (34). Monocytes treated with 20 \mu M nigericin in the NaCl-based minimal medium released large quantities of radiolabeled 17-kDa IL-1\textbeta (Fig. 7, lanes 1 and 2). In contrast, monocytes that were treated with nigericin while maintained in the 137 mM choline chloride- or the 250 mM sucrose-based media did not produce 17-kDa IL-1\textbeta (Fig. 7, lanes 3, 4, 7, and 8). A minimal medium composed of 37 mM NaCl and 100 mM choline chloride allowed 17-kDa IL-1\textbeta production in response to nigericin (Fig. 7, lanes 5 and 6), but the quantities of mature cytokine generated were less than that produced by cells maintained in the presence of 137 mM NaCl.

Further evidence that Na\textsuperscript{+} is required for a step downstream of the P\textsubscript{2z} receptor was obtained by examining release of the K\textsuperscript{+} analog \[^{86}\text{Rb}^+\] from ATP-treated cells. Ligation of the P\textsubscript{2z} receptor is known to promote membrane depolarization and loss of intracellular K\textsuperscript{+} (44). Monocytes were loaded with \[^{86}\text{Rb}^+\], then its efflux was measured in the absence and presence of ATP. In the absence of ATP, the isotope slowly dissociated from monocytes, and this was independent of whether the cells were maintained in an NaCl- or a choline chloride-based medium (Fig. 8). Addition of ATP greatly accelerated the rate of \[^{86}\text{Rb}^+\] efflux (Fig. 8) in Na\textsuperscript{+}-containing and Na\textsuperscript{+}-depleted media (Fig. 8). The initial rate of release was augmented in the choline chloride medium, but after 10 min of ATP exposure a similar overall percentage of \[^{86}\text{Rb}^+\] was released from cultures maintained in the absence or presence of Na\textsuperscript{+} (Fig. 8).

**DISCUSSION**

Results of these studies indicate that extracellular monovalent cations serve as important regulators of stimulus-coupled IL-1\textbeta posttranslational processing. Replacement of medium Na\textsuperscript{+} with Li\textsuperscript{+}, K\textsuperscript{+}, or choline inhibited ATP-induced formation of 17-kDa IL-1\textbeta. Likewise, when the extracellular ionic concentration was reduced by employing a medium containing 0.25 M sucrose, LPS-activated monocytes failed to produce extracellular 17-kDa IL-1\textbeta in response to ATP stimulation. The various media replacements, however, did not yield equivalent patterns of inhibition. Monocytes maintained in a choline chloride- or sucrose-based medium responded to ATP as evidenced by release of pro-IL-1\textbeta, but less total IL-1\textbeta was externalized relative to cells maintained in a minimal NaCl medium. Importantly, exchange of Na\textsuperscript{+} for choline restored formation and release of mature IL-1\textbeta; partial processing was observed at 45 mM Na\textsuperscript{+}, and a near-normal processing capacity was obtained with 92 mM extracellular Na\textsuperscript{+}. Likewise, titration of Na\textsuperscript{+} into the sucrose medium restored processing; yield of extracellular 17-kDa IL-1\textbeta under these conditions often exceeded that produced in the minimal NaCl-based medium. The enhanced yield of mature cytokine likely reflects the reduction in extracellular Cl\textsuperscript{-}; we previously observed that cells maintained in 137 mM sodium gluconate yielded greater levels of 17-kDa IL-1\textbeta than cells maintained in 137 mM NaCl (34). An accentuated outward Cl\textsuperscript{-} gradient may favor the ATP response. Because Na\textsuperscript{+} concentrations required to restore cytokine processing were similar in choline- and sucrose-based minimal media, it is the absence of Na\textsuperscript{+}, rather than the presence of choline or sucrose, that is considered responsible for the inhibitory effects.
Substitution of K\(^+\) for Na\(^+\) within the medium yielded a different outcome. Cells maintained in a 137 mM KCl-based medium responded to ATP and released pro-IL-1β. Backexchange with Na\(^+\) ultimately led to formation of the 17-kDa extracellular cytokine, but return of the cytokine response did not correspond with the aforementioned Na\(^+\) requirements. Thus, when the medium contained 100 mM Na\(^+\) and 37 mM K\(^+\), production of 17-kDa IL-1β was inhibited by 90%. Because 92 mM Na\(^+\) was sufficient to support a complete stimulus-coupled IL-1β posttranslational processing response in the presence of 45 mM choline, the lingering inhibition observed in the presence of 37 mM extracellular K\(^+\) must reflect the elevated level of this cation. Reducing the K\(^+\) concentration to 4.2 mM (levels found in the basal NaCl medium) allowed normal stimulus-coupled IL-1β posttranslational processing. Previously, the impaired cytokine response observed after complete replacement of Na\(^+\) with K\(^+\) was interpreted as evidence that high extracellular K\(^+\) prevented depletion of this cation from intracellular pools and, in turn, blocked cytokine posttranslational processing (32, 47). The present observation indicating that Na\(^+\) depletion inhibits stimulus-coupled processing complicates this interpretation. However, because cytokine posttranslational processing remained impaired when the medium contained elevated K\(^+\) (37 mM) and sufficient extracellular Na\(^+\) (100 mM) to support the ATP response, the notion that depletion of intracellular K\(^+\) stores is necessary for efficient cytokine posttranslational processing remains valid. The ability of K\(^+\) ionophores and hypotonic stress, two treatments that mobilize intracellular K\(^+\), to serve as alternate triggers of IL-1 posttranslational processing provides additional evidence that K\(^+\) depletion serves as a key element of the cytokine response pathway (32, 34, 47). It is interesting to note that K\(^+\) depletion also has been demonstrated to be a necessary component of the apoptotic response (17). The similar requirement for K\(^+\) efflux supports the notion that cytokine release is associated with a form of programmed cell death (15).

Monocytes maintained in a 137 mM LiCl-based medium also demonstrated an aberrant ATP responsiveness. Cells maintained in this medium released pro-IL-1β in response to ATP, and the total quantity of IL-1β externalized was comparable to that released by cells maintained in an NaCl-based medium. Titration of Na\(^+\) for Li\(^+\) led to decreased extracellular pro-IL-1β levels, but in the presence of ≥45 mM Li\(^+\) no 17-kDa IL-1β was produced. Because this inhibition by Li\(^+\) was observed in the presence of Na\(^+\)-concentrations (92 mM) sufficient to support normal processing, Li\(^+\) appears to be inhibitory. How Li\(^+\) inhibits this response is unclear, but these cations are known to affect activity of a variety of cellular components, including protein kinases (26) and membrane transporters (21).

How may Na\(^+\) affect stimulus-coupled IL-1β posttranslational processing? Since neither 5-N,N-diethylamiloride nor ouabain inhibited ATP-induced processing, the Na\(^+\)/H\(^+\) antiporter and the Na\(^+\)-K\(^+\)-ATPase do not appear to be the targets of the Na\(^+\) effect. ATP binding to macrophage surface P\(_{2\alpha}\) purinoceptors leads to major changes in the intracellular ionic environment, resulting in a complete depolarization of the membrane potential (4, 44). Mouse thymocytes and human sperm also are reported to depolarize when treated with millimolar concentrations of ATP (10, 36). When these latter cell types were treated with ATP in the absence of extracellular Na\(^+\), however, the extent of depolarization was suppressed (10, 36). Influx of extracellular Na\(^+\) through an ATP-induced cation channel may be required to achieve complete membrane depolarization, and/or Na\(^+\) may be required to keep the ATP-induced channel in an open conformation. The latter possibility seems unlikely, since monocytes treated with ATP in the absence of Na\(^+\)-released 

Additional evidence that the Na\(^+\)-requiring step is unrelated to caspase-1 activation stems from the observation that monocytes released pro-IL-1β via an ethacrynic acid-inheritable process in the presence of Na\(^+\) and a caspase inhibitor but released the procycokine in an ethacrynic acid-insensitive manner in the absence of
Na⁺. If Na⁺ depletion simply inhibited caspase-1, then export of pro-IL-1β in the presence of YVAD-CHO would be expected to mimic export in Na⁺-deficient medium. Therefore, Na⁺ appears to be required at a step in the IL-1β posttranslational pathway that precedes caspase-1 activation and cytokine externalization. Lack of external Na⁺ recently was reported to impair release of Ca²⁺ from intracellular stores in response to ATP stimulation of rat parotid acinar cells (11). Perhaps a rise in intracellular Ca²⁺ is needed for the monocyte response. Additional studies are required to clarify the Na⁺ dependence of this novel cytokine posttranslational processing.

Despite their inability to produce mature IL-1β, monocytes treated with ATP in the absence of extracellular Na⁺ released elevated levels of unprocessed procytokine. This ATP-induced, Na⁺-independent export occurred via a mechanism distinct from that employed to release 17-kDa IL-1β in the presence of Na⁺ on the basis of 1) the differential rate of IL-1β export between the two processes and 2) the differential sensitivity of the two processes to ethacrynic acid. We suspect in the absence of Na⁺ that ATP promotes an osmotic imbalance within monocytes that leads to swelling and lysis. Changes to the intracellular ionic environment that occur in the absence of Na⁺, however, are not sufficient or occur in an inappropriate order such that caspase-1 fails to activate. As a result, pro-IL-1β is not converted to the 17-kDa species. The inability of ethacrynic acid to suppress ATP-induced release of pro-IL-1β in the absence of Na⁺ is consistent with the notion that the nonproteolytically processed species is released via an aberrant (lytic) mechanism that is insensitive to pharmacological intervention.

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