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# Kinetics and Mechanism of ATP-Dependent IL-1 $\beta$ Release from Microglial Cells<sup>1</sup>

Juana M. Sanz and Francesco Di Virgilio<sup>2</sup>

Endotoxin-dependent release of IL-1 $\beta$  from mouse microglial cells is a very inefficient process, as it is slow and leads to accumulation of a modest amount of extracellular cytokine. Furthermore, secreted IL-1 $\beta$  is mostly in the procytokine unprocessed form. Addition of extracellular ATP to LPS-primed microglia caused a burst of release of a large amount of processed IL-1 $\beta$ . ATP had no effect on the accumulation of intracellular pro-IL-1 $\beta$  in the absence of LPS. In LPS-treated cells, ATP slightly increased the synthesis of pro-IL-1 $\beta$ . Optimal ATP concentration for IL-1 $\beta$  secretion was between 3 and 5 mM, but significant release could be observed at concentrations as low as 1 mM. At all ATP concentrations IL-1 $\beta$  release could be inhibited by increasing the extracellular K<sup>+</sup> concentration. ATP-dependent IL-1 $\beta$  release was also inhibited by 90 and 60% by the caspase inhibitors YVAD and DEVD, respectively. Accordingly, in ATP-stimulated microglia, the p20 proteolytic fragment derived from activation of the IL-1 $\beta$ -converting enzyme could be detected by immunoblot analysis. These experiments show that in mouse microglial cells extracellular ATP triggers fast maturation and release of intracellularly accumulated IL-1 $\beta$  by activating the IL-1 $\beta$ -converting enzyme/caspase 1. *The Journal of Immunology*, 2000, 164: 4893–4898.

Interleukin-1 is a potent proinflammatory cytokine that is likely involved in several disease conditions such as chronic inflammations, septic shock, acute myelocytic leukemia, and cachexia (1–3). Biological activity of IL-1 is the result of transcription of two related gene products: IL-1 $\alpha$  and IL-1 $\beta$ . Both IL-1 $\alpha$  and IL-1 $\beta$  are synthesized as 31–34-kDa procytokines and are then converted into the mature 17-kDa form; however, although IL-1 $\alpha$  is biologically active in both the pro and mature form, IL-1 $\beta$  is only active in the mature form (4). Conversion of pro into mature IL-1 $\beta$  is catalyzed by a cysteine protease known as IL-1 $\beta$ -converting enzyme (ICE)<sup>3</sup> (3) that is the prototypical member of the caspase family (caspase 1) (5, 6). Proteolytical maturation is also linked via a poorly known mechanism to IL-1 $\beta$  release into the extracellular space. LPS is an efficient stimulus for IL-1 $\beta$  release from circulating monocytes, but when these cells are cultured in vitro for a few days, they become refractory to LPS stimulation (7, 8). Likewise, peritoneal macrophages, microglial cells, and dendritic cells respond weakly to LPS stimulation, thus suggesting that a second stimulus may be needed to elicit IL-1 $\beta$  secretion from these cell types.

Among stimuli reported to enhance and accelerate IL-1 $\beta$  release from mononuclear phagocytes (cytolytic T cells, K<sup>+</sup> ionophores, bacterial exotoxins, and ATP) (7–10), ATP is one of the most interesting because this nucleotide is present at a concentration of

5–10 mM in the cytosol of most cells, thus it can be released in large amounts following plasma membrane damage or acute cell death. Furthermore, it is becoming increasingly clear that ATP is released by several cell types via nonlytic pathways in response to stimulation with many different agonists, among which is LPS itself (11–15). Once in the pericellular environment, ATP acts at plasma membrane P2 purinergic receptors, thus leading to cell activation. Mononuclear phagocytes express to high level a member of the P2X subfamily named P2X<sub>7</sub> (also previously known as P2Z) that, upon ligation by ATP, mediates large transmembrane cation fluxes and, under extreme conditions of activation, the formation of nonselective plasma membrane pores (16–18). As reported by several laboratories, ATP, via activation of P2X<sub>7</sub>, is one of the most powerful stimuli for secretion of IL-1 $\beta$  in its mature form (10, 19–22). Rather interestingly, ATP is an efficient stimulus for IL-1 $\beta$  secretion only after the cells have undergone a short priming with endotoxin, suggesting that this nucleotide is unable to trigger transcription of the IL-1 $\beta$  gene, but rather acts posttranslationally, probably accelerating the step(s) involved in the proteolytical maturation of this cytokine.

It was originally proposed by us and others that ATP-mediated IL-1 $\beta$  processing in macrophages and microglia is due to activation of ICE or a related caspase (8, 20, 23), but direct proof has not yet been provided. In the present work, we have investigated the mechanism of ATP-dependent IL-1 $\beta$  release in mouse microglia. Our findings suggest that extracellular ATP, acting at the P2X<sub>7</sub> receptor, triggers ICE activation and provides evidence for a crucial role of changes in the cytoplasmic K<sup>+</sup> concentration as the coupling factor.

## Materials and Methods

### Cells and solutions

Microglial N13 cells were obtained as described by Righi et al. (24) and cultured in RPMI 1640 medium supplemented with (Sigma, St. Louis, MO) 2 mM glutamine, 10% heat-inactivated FCS (Life Technologies Ltd., Paisley, Scotland), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Experiments were conducted in this culture medium without FCS. LPS (Sigma) was always used at a concentration of 1  $\mu$ g/ml.

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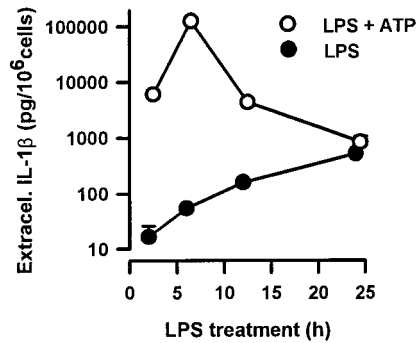
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<sup>3</sup> Abbreviation used in this paper: ICE, IL-1 $\beta$ -converting enzyme.



**FIGURE 1.** Kinetics of IL-1 $\beta$  release triggered by LPS or LPS plus ATP. Microglial cells were plated in 24-well plates in RPMI at a concentration of  $5 \times 10^5$ /well and primed with LPS for 2, 6, 12, or 24 h. After this time, supernatants were collected to measure the amount of IL-1 $\beta$  released in response to LPS alone (●) or the incubation was prolonged for 30 additional minutes in the presence of 5 mM ATP (○) to measure stimulation of IL-1 $\beta$  release triggered by P2X<sub>7</sub> receptor activation.

#### Measurement of enzymatic activity and IL-1 $\beta$ release

Lactic dehydrogenase activity was measured as described previously (25). Intracellular and extracellular IL-1 $\beta$  were measured with the same Endogen mouse IL-1 $\beta$  ELISA kit (Endogen, Woburn, MA) that does not discriminate between the pro and mature cytokine forms. The caspase 1/ICE inhibitor YVAD-CHO and the caspase 3/apopain inhibitor DVAD-CHO were purchased from Bachem Feinchemikalen AG (Bubendorf, Switzerland).

#### Immunoblotting and caspase 1/ICE activation

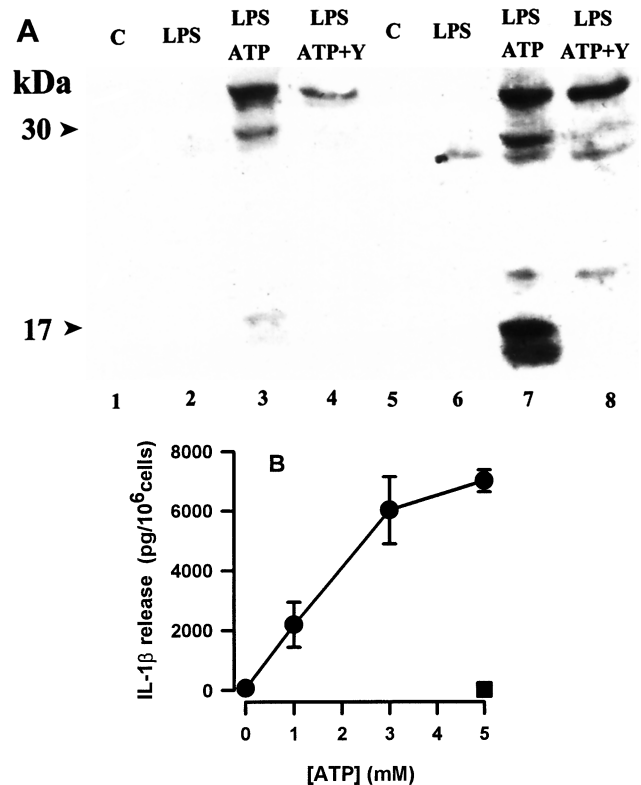
For IL-1 $\beta$  detection, cells were lysed with 0.1% Triton X-100 solution in PBS. Cell lysates and supernatants were run on a 12% polyacrylamide gel (Merck, Milan, Italy) and blotted onto a reinforced nitrocellulose filter (Amersham International, Amersham, U.K.). IL-1 $\beta$  was detected with a goat anti-mouse IL-1 $\beta$  polyclonal Ab (Genzyme, Cinisello Balsamo, Italy) followed by staining with protein A labeled with HRP and visualization by chemiluminescence (Amersham). For caspase 1 detection, cell extracts were prepared by resuspending PBS-washed cell pellets in a high-salt buffer solution containing 20 mM HEPES (pH 7.9), 350 mM NaCl, 20% glycerol, 1% IGEPAL CA-630 (Sigma), 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 2 mM PMSF, and 2  $\mu$ g/ml aprotinin. Extracts were incubated on ice for 20 min and then cleared by centrifugation. Supernatants were run on a 15% polyacrylamide gel. The p20 caspase 1 proteolytic fragment was detected with peroxidase-conjugated affinity-purified goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Caspase 1/ICE activity was also measured with a fluorometric assay based on the cleavage of the YVAD-7-amino-4-trifluoromethyl coumarin (YVAD-AFC) specific caspase 1 substrate (Medical Biological Laboratories, Nagoya, Japan).

#### Data presentation

Data shown are averages  $\pm$  SD of determinations from three to four experiments performed in duplicate. Sometimes error bars are not shown because their width exceeded that of the symbol.

## Results

It is well documented that endotoxin by itself is an inefficient stimulus for IL-1 $\beta$  release from microglial cell lines as well as from primary microglia cultures in vitro (19, 23). Fig. 1 reports an experiment in which N13 cells were stimulated with LPS (filled circles) and the supernatants withdrawn for IL-1 $\beta$  determination at the indicated time points. In a parallel experiment, the cultures were briefly pulsed for 30 min with ATP (open circles) after the increasing intervals of LPS treatment. The supernatants were then withdrawn for IL-1 $\beta$  measurement. Addition of ATP after a 2-h pretreatment with LPS increased accumulation of extracellular IL-1 $\beta$  from about 20 to 5000 pg/10<sup>6</sup> cells. Even more impressive was the enhancement (from 100 to 100,000 pg/10<sup>6</sup> cells) observed

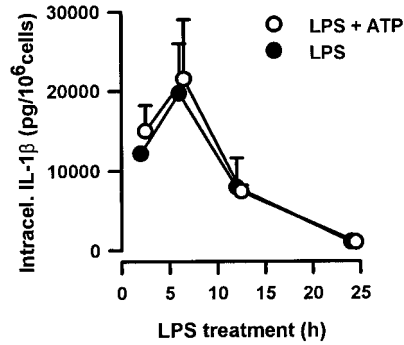


**FIGURE 2.** Extracellular ATP causes release of mature IL-1 $\beta$ . **A**, Microglial cells were pretreated with LPS for 2 h (lanes 2–4) or 6 h (lanes 6–8). In lanes 3 and 7, 5 mM ATP was added for 30 min. In lanes 4 and 8, 50  $\mu$ M YVAD-CHO was added before 5 mM ATP. Lanes 1 and 5, control cells. Supernatants from  $2 \times 10^6$  cells were withdrawn, concentrated by filtration through Millipore Ultrafree filters (Bedford, MA) with a 10,000-Da nominal molecular mass cutoff, and separated by SDS-gel electrophoresis. **B**, Cells ( $5 \times 10^5$ /ml) were pretreated for 2 h with LPS, stimulated with ATP for 30 min (●), and supernatants were withdrawn for IL-1 $\beta$  determination. ■, IL-1 $\beta$  release from samples incubated with ATP in the absence of added LPS.

when ATP was added 6 h after LPS. The ability of ATP to stimulate IL-1 $\beta$  release decreased when the interval after LPS addition was extended over 6 h, and after 24 h any stimulatory activity was completely lost.

LPS by itself not only released little IL-1 $\beta$ , but also almost exclusively of the uncleaved, 34-kDa form, even after a 6-h incubation (Fig. 2A, lane 6). ATP, on the contrary, promoted efficient cleavage of the cytokine when added to cells that had been preincubated in the presence of LPS (Fig. 2A, lanes 3 and 7), although the amount of the 17-kDa form was much larger in the supernatants of microglial cells treated with LPS for 6 h (Fig. 2A, lane 7) compared with those treated for 2 h (lane 3). A short 5-min incubation with the ICE/caspase 1 inhibitor YVAD before the addition of ATP completely inhibited the appearance of the 17-kDa band (lanes 4 and 8) and also greatly decreased the release of the 34-kDa band and other proteolytic fragments. The inhibitory effect of YVAD was more marked when cells were primed with LPS for 2 h compared with a 6-h priming (cf lanes 4 and 8). LPS by itself, applied for 2 or 6 h, triggered no IL-1 $\beta$  release whether in the 34- or 17-kDa forms (lanes 2 and 6).

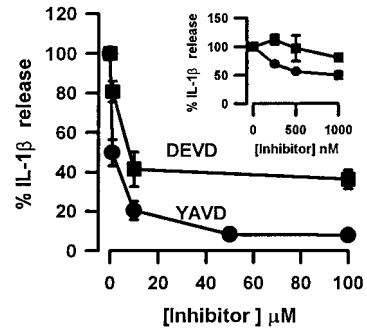
The ATP dose-dependency for IL-1 $\beta$  release (Fig. 2B) showed a plateau at a concentration of about 3 mM, in agreement with previous findings in mouse microglia (20), but slightly higher than previously reported by our laboratory for human macrophages (21).



**FIGURE 3.** Lack of effect of ATP on intracellular IL-1 $\beta$  accumulation. Microglial cells were plated and stimulated as reported in Fig. 1. Supernatants were withdrawn and discarded, the cell monolayers were extensively rinsed with fresh medium and lysed with Triton X-100 (0.1% in RPMI), and then the IL-1 $\beta$  content of the cell lysates was measured.

We then measured the kinetics of accumulation of intracellular IL-1 $\beta$  in cells treated with LPS or LPS plus ATP (Fig. 3). LPS caused a maximal increase in the intracellular IL-1 $\beta$  content 6 h after stimulation and then cytokine content declined to reach nearly undetectable levels after 24 h. Stimulation with ATP for 30 min slightly but consistently enhanced the amount of intracellular IL-1 $\beta$ , whether added 2 or 6 h after LPS, but this small increase was not statistically significant. A comparison between Figs. 1 and 3 suggests that there is a close correlation between the amount of the intracellularly available IL-1 $\beta$  and the amount that can be released by ATP, since peak release by this nucleotide was achieved 6 h after pretreatment with LPS, when availability of intracellular IL-1 $\beta$  was maximal and the lowest ATP-mediated release occurred after 24 h of LPS incubation, when intracellular IL-1 $\beta$  was almost undetectable. We would expect to find a decrease of intracellular IL-1 $\beta$  in the ATP-treated cells, as a consequence of the large secretion triggered by this nucleotide, but to our surprise we found no change or, at the most, a small increase, as if ATP-stimulated cells not only released more IL-1 $\beta$ , but also accumulated more procytokine, secretion being probably the rate-limiting step for further procytokine synthesis. If this were the case, i.e., if ATP-dependent IL-1 $\beta$  release depended in part on the continuous neosynthesis of pro-IL-1 $\beta$ , then it should be at least in part sensitive to inhibition by blockers of protein synthesis. This is indeed the case as addition of cycloheximide before ATP reduced intracellular and extracellular IL-1 $\beta$  accumulation by about 15 and 30%, respectively, compared with samples treated with ATP in the absence of the inhibitor (Table I).

The mechanism by which ATP causes maturation and release of mature IL-1 is not completely understood, but it likely involves activation of ICE/caspase 1. This hypothesis is supported by the experiment reported in Fig. 4 showing that the ICE/caspase 1-selective blocker YVAD inhibited IL-1 $\beta$  release with an IC<sub>50</sub> of



**FIGURE 4.** Effect of caspase inhibitors on ATP-dependent IL-1 $\beta$  release. Cells were plated in 24-well plates at a concentration of  $5 \times 10^5$ /ml for 2 h, incubated with increasing concentrations of YVAD-CHO or DEVD-CHO, and stimulated for 30 min with 5 mM ATP. Supernatants were then withdrawn and IL-1 $\beta$  was measured. Inset, An expansion of the dose-dependency curve at very low inhibitor concentrations. IL-1 $\beta$  release is shown as pg/10<sup>6</sup> cells.

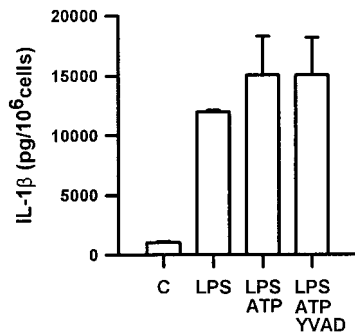
about 1  $\mu$ M, whereas the less selective tetrapeptide DEVD was much less active (IC<sub>50</sub>, 10  $\mu$ M), and even at high concentrations was unable to fully prevent IL-1 $\beta$  release. To further support a selective effect of YVAD on ICE/caspase 1 processing, Fig. 5 shows that this tetrapeptide had no effect on the accumulation of pro-IL-1 $\beta$ .

ICE/caspase 1 is synthesized as a 40-kDa precursor that needs to be proteolytically activated to process pro-IL-1 $\beta$ , but the mechanism of activation is unknown. Over the last few years, several reports in monocyte/macrophage cells have suggested that a decrease in the intracellular K<sup>+</sup> concentration could be the trigger for ICE/caspase 1 activation. Fig. 6 suggests that this is also likely the case in microglia since it shows that increasing the external KCl content of a normal RPMI medium strongly inhibited ATP-dependent IL-1 $\beta$  release, although under these ionic conditions P2X<sub>7</sub> receptor activation was not inhibited (data not shown). The reduced cytokine release was not due to hypertonicity because the addition of NaCl had no or very little effect. Further proof of the ability of ATP to turn on ICE/caspase 1 comes from the detection of the p20 ICE/caspase 1 fragment in ATP-challenged cells (Fig. 7). Although some p20 accumulation was detectable in cells treated with ATP alone (lane 3), an unequivocal band was clearly detected only in the sample that was treated with LPS before ATP addition (lane 2), suggesting that LPS treatment also increased synthesis of the p45 ICE/caspase 1 precursor. Treatment with LPS in the absence of ATP caused no p20 fragment formation (data not shown). We confirmed activation of ICE/caspase 1 by ATP by measuring the cleavage of the fluorogenic substrate YVAD-7-amino-4-trifluoromethyl coumarin (YVAD-AFC) (Fig. 8). Cell lysates of control or ATP-treated samples were incubated in the presence of the fluorogenic substrate for increasing times (1–3 h), clearly showing that although control samples (open circles) produced a

Table I. Effect of cycloheximide on IL-1 $\beta$  accumulation<sup>a</sup>

Addition	Intracellular IL-1 $\beta$ , (pg/10 <sup>6</sup> )	Extracellular IL-1 $\beta$ , (pg/10 <sup>6</sup> )	Total IL-1 $\beta$ , (pg/10 <sup>6</sup> )
ATP	40,646 $\pm$ 2,040	72,886 $\pm$ 1,011	113,530 $\pm$ 12,155
ATP + cycloheximide	34,955 $\pm$ 4,000	48,833 $\pm$ 1,200	85,147 $\pm$ 8,200

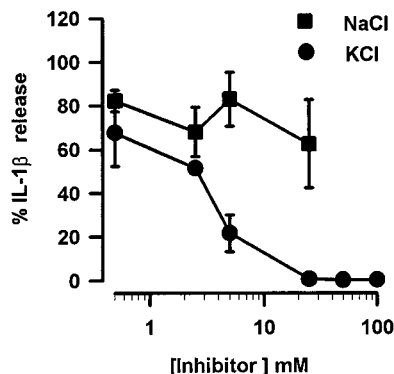
<sup>a</sup> Microglial cells were plated in 24-well plates in RPMI at a concentration of  $5 \times 10^5$ /well and primed with LPS for 6 h before stimulation of 5 mM ATP for 30 min in the absence and presence of 50  $\mu$ M cycloheximide (added 5 min before ATP). At the end of the incubation, supernatants were withdrawn and monolayers were extensively rinsed with fresh RPMI before lysis. IL-1 $\beta$  was measured separately in the supernatants and in the cell lysates. Values are averages  $\pm$  SD of triplicate determinations from one experiment representative of three similar experiments.



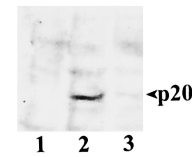
**FIGURE 5.** Lack of effects of YVAD on intracellular IL-1 $\beta$  accumulation. Cells were plated as described in Fig. 2 and then primed for 2 h with LPS followed by either 5 mM ATP or 50  $\mu$ M YVAD-CHO plus 5 mM ATP. Supernatants were discarded, monolayers were thoroughly rinsed and lysed with a 0.1% Triton X-100 solution in RPMI, and the lysates were used to measure IL-1 $\beta$ .

negligible increase in fluorescence, the ATP-treated samples (open triangles) generated at least a 3-fold increase in fluorescence. It is of interest that the mere treatment with LPS (open squares) caused no caspase 1/ICE activation.

Fig. 9 compares the kinetics of accumulation of intracellular and extracellular IL-1 $\beta$  in the presence of LPS (Fig. 9A) or LPS plus ATP (Fig. 9B). By comparing intracellular and extracellular IL-1 $\beta$  in microglia stimulated with LPS alone, it can be observed that a very small fraction of the total cellular cytokine was released (e.g., 6 h after LPS, 50 pg/10<sup>6</sup> cells was found extracellularly against about 20,000 pg/10<sup>6</sup> cells found intracellularly); furthermore, after an initial phase during which intracellular and extracellular IL-1 $\beta$  accumulated in parallel, intracellular IL-1 $\beta$  declined while extracellular accumulation showed a steady rate of increase. However, even at the point of maximal release (24 h), extracellular IL-1 $\beta$  was only a very small fraction of the total, a finding that further supports the observation that LPS is a very inefficient stimulus for secretion and that, in the absence of release, most of the intracellular cytokine is degraded. In the samples treated with LPS plus ATP, intracellular accumulation and release of IL-1 $\beta$  were more closely related (Fig. 9B). Maximal release occurred when ATP was added 6 h after LPS. At later time points, secretion showed a fast



**FIGURE 6.** Dose dependency and K<sup>+</sup> inhibition of IL-1 $\beta$  release. Cells ( $5 \times 10^5$ /ml) were plated in 24-well plates in RPMI and primed with LPS for 2 h. Right before the stimulation with ATP (5 mM), increasing concentrations of either NaCl (■) or KCl (●) were added to the samples. Supernatants were withdrawn for IL-1 $\beta$  measurement after 30 min. Values are expressed as percentage of release triggered by ATP in control samples incubated in RPMI with no further salt added. IL-1 $\beta$  release is shown as pg/10<sup>6</sup> cells.



**FIGURE 7.** Extracellular ATP causes caspase 1 (ICE) cleavage. Microglial cells ( $2.5 \times 10^6$ /well) were incubated for 6 h in LPS-supplemented RPMI and then stimulated for 30 min with 5 mM ATP (lane 2) or with 5 mM ATP without prior LPS treatment (lane 3). Lane 1, Control cells incubated for 6 h in RPMI alone.

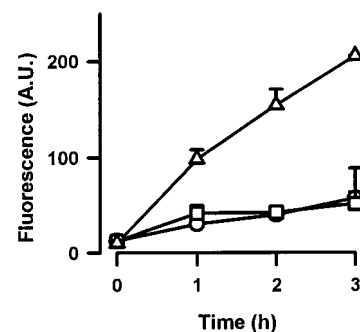
decline, presumably reflecting exhaustion of the intracellular IL-1 $\beta$  reservoir.

## Discussion

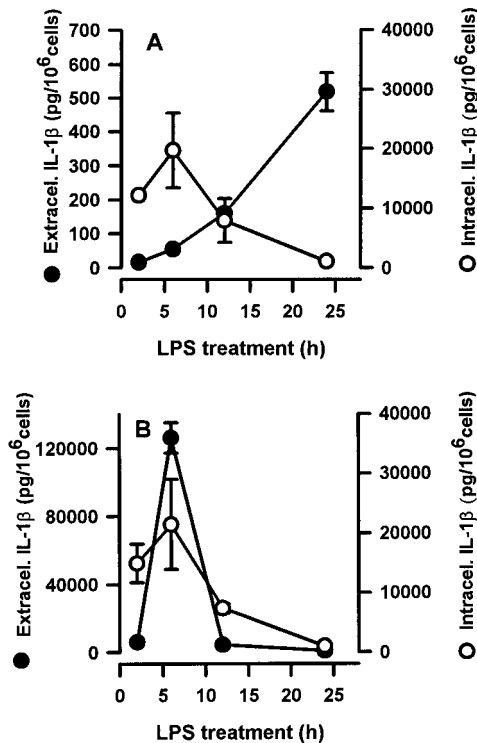
It has been shown by several groups that extracellular ATP is a powerful stimulus for release of IL-1 $\beta$  in its processed form from macrophages and microglial cells (10, 19–23). The receptor involved is the P2X<sub>7</sub> purinoceptor, a plasma membrane receptor formed by an unknown number of subunits each 595 aa long (17, 18). P2X<sub>7</sub> is an unusual, bifunctional receptor that upon transient stimulation with ATP behaves as a typical cation-selective ion channel permeable to K<sup>+</sup>, Na<sup>+</sup>, and Ca<sup>2+</sup> (14–16). However, upon repetitive stimulation, it undergoes a transition into a nonselective pore that allows transmembrane fluxes of hydrophilic molecules up to 900 Da. So far, there has been no report of any known intracellular second messengers being generated upon P2X<sub>7</sub> activation, besides the obvious increase in cytosolic Ca<sup>2+</sup>, nor P2X<sub>7</sub> is known to interact with cytoplasmic proteins, thus the ability to cause IL-1 $\beta$  processing and release is not easy to explain.

IL-1 $\beta$  is synthesized as a 31–34-kDa procytokine that is converted into the mature 17-kDa biologically active form by a cysteine protease named ICE (1–4), also known as caspase 1, this enzyme being the founder member of the caspase family. ICE/caspase 1 itself is synthesized as a 45-kDa precursor (p45) that is then proteolytically cleaved into the active (p10/p20)<sub>2</sub> form (5). The enzyme(s) responsible for the cleavage of caspase 1 is unknown; however, since this cysteine protease is capable of auto-processing, activation might be due to an autoproteolytic process.

It was initially reported by Perregaux and Gabel (10) and Walev et al. (26) that maneuvers aimed at reducing the intracellular K<sup>+</sup>



**FIGURE 8.** Extracellular ATP causes caspase 1 (ICE) activation. Microglial cells ( $2.2 \times 10^6$ ) were incubated for 6 h with either LPS alone (□) or LPS plus 5 mM ATP (30 min,  $\Delta$ ). ○, Controls. After this incubation time, samples were rinsed, lysed, and used to measure caspase 1/ICE activity according to the manufacturer's indications. Cell lysates were incubated in the presence of the fluorogenic substrate for increasing times, as shown on the abscissa. Fluorescence increase is shown as arbitrary units (A.U.).



**FIGURE 9.** Effect of LPS and ATP on the accumulation of intracellular and extracellular IL-1 $\beta$ . Cells were processed as in Fig. 1. In *A*, cells were stimulated for the indicated times with LPS alone. In *B*, cells were stimulated with LPS for the indicated times plus 5 mM ATP (30 min).

concentration, either by means of selective K<sup>+</sup> ionophores or inhibitors of plasma membrane K<sup>+</sup> channels, caused strong activation of IL-1 $\beta$  release in its mature form. However, it was not until 1998 that formal demonstration was provided that reducing the K<sup>+</sup> concentration led to ICE/caspase 1 cleavage (27). The activatory effect of low K<sup>+</sup> could also be shown on the isolated recombinant enzyme. These observations raise an obvious question: assuming that the activatory pathway involving a reduction in intracellular K<sup>+</sup> has a physiological relevance in ICE/caspase 1 activation, what is the natural agonist that may cause cellular K<sup>+</sup> efflux, given that neither nigericin nor K<sup>+</sup> channel blockers are expected to be physiologically present in the body? Extracellular ATP is clearly an interesting candidate to such a role.

It is now well ascertained that ATP functions as an extracellular messenger in the nervous system as well as in the gut, skin, cardiovascular apparatus, and immune system (28–30). This nucleotide can be released by different mechanisms such as secretory exocytosis, plasma membrane transporters, passive leakage across the plasma membrane, or massive efflux as a consequence of cell death (14, 31–34). This latter mechanism might be very relevant for IL-1 $\beta$  secretion since it is well known that cell damage is an important cause of inflammation and vice versa. Therefore, it is not unlikely that a significant concentration of extracellular ATP could build up at inflammatory sites. Besides passive leakage, some inflammatory cells are capable of actively releasing ATP in response to extracellular stimulation. Sitkovsky's laboratory (35) has shown that up to 15% of total cellular ATP can accumulate in the pericellular environment in response to stimulation of CTL with anti-CD3 or anti-TCR Abs. We and others have shown that macrophages and microglial cells secrete ATP when stimulated with LPS (14, 15, 36), not to mention platelets that store massive amounts of ATP within dense granules (37), and can therefore

cause significant increases in the extracellular ATP concentration when activated. That inflammatory cells are physiologically exposed to extracellular ATP is also hinted to by the expression of ATP-hydrolyzing enzymes, such as ecto-diphosphohydrolase (ecto-apyrase) and ecto-ATPase, on the outer aspect of their plasma membrane (38, 39).

Altogether, controlled release of ATP, presence of specific ATP receptors, and powerful ATP-consuming systems points to the operation of an ATP-based autocrine/paracrine loop that might have an important role in the local modulation of inflammation (40). ATP has long been suspected to be involved in the short range modulation of inflammatory cells, but it has always been difficult to pinpoint a unique ATP-dependent response. Control of IL-1 $\beta$  maturation and secretion is a good candidate function for extracellular ATP.

LPS, the best known stimulus for IL-1 $\beta$  release from macrophages and microglial cells, is very inefficient *in vitro* because it causes little secretion and mostly in the 34-kDa unprocessed form. Thus, it is postulated that a second factor that triggers ICE/caspase 1 activation is needed. Although, on one hand, this two-step process reveals the existence of tightly controlled mechanisms for IL-1 $\beta$  secretion, on the other it raises the question of the identity of the ICE/caspase 1-activating factor. Posttranslational IL-1 $\beta$  processing has been achieved by treating LPS-primed mononuclear phagocytes with several factors: nigericin, cytolytic toxins, cytolytic T cells, and ATP (8, 9, 19, 20). Among them all, ATP is the only agent likely to have a widespread physiological role, since nigericin is an experimental tool, cytolytic toxins will only be present during bacterial sepsis, and, finally, *in vivo* generation of anti-macrophage cytolytic T cells is an unusual event. Thus, ATP could be the external trigger for the proteolytic cleavage and externalization of pro-IL-1 $\beta$  accumulated intracellularly upon LPS stimulation. The data reported in the present work provide further support for the role of K<sup>+</sup> in the coupling between P2X<sub>7</sub> and ICE/caspase 1. ATP, via activation of P2X<sub>7</sub>, is well known to cause a large K<sup>+</sup> efflux (10) and thus a drop in the cytoplasmic concentration of this cation. We wonder whether also the other biological agents capable of triggering IL-1 $\beta$  maturation (e.g., bacterial toxins and cytolytic lymphocytes) might act through this same mechanism, since they also are membrane-perturbing agents. This interpretation is supported by the inhibitory effect on IL-1 $\beta$  release observed by raising extracellular K<sup>+</sup>, a maneuver that prevents movement of this cation along its chemical gradient (outwardly directed), as shown earlier by Perregaux and Gabel (10). The study by Cheneval et al. (27) performed in a human monocyte cell line lends further support to this hypothesis by showing that enzymatic activity of recombinant ICE/caspase 1 also requires a drop in the K<sup>+</sup> concentration. It is likely that in the intact cell, P2X<sub>7</sub>-dependent changes in cytoplasmic K<sup>+</sup> will be quickly transmitted to ICE/caspase 1 since this enzyme is thought to be compartmentalized in the subplasmalemmal cytoplasm.

In the absence of ATP, the kinetics of IL-1 $\beta$  accumulation and release from microglial cells closely matched that previously described in murine macrophages (7). Peak intracellular accumulation occurred 6 h after LPS addition, then a steady decline followed. Externalization was much slower, never reaching >3% of total (intracellular plus extracellular) IL-1 $\beta$ , in agreement with previous data (7). In the presence of ATP, efficiency of the release process was dramatically enhanced. When added 2 h after LPS, ATP triggered the release of about 25% of the total cellular IL-1 $\beta$  content (5,000 vs 20,000 pg/10<sup>6</sup> cells, respectively); however, when added 6 h after LPS, ATP released >80% of total IL-1 $\beta$  (125,000 vs 145,000 pg/10<sup>6</sup> cells, respectively). Acceleration of release was not due to an increased rate of cell death because, as

previously shown (21, 22), for these short incubations ATP has no or minimal cytotoxic effect.

The ICE/caspase 1 inhibitor YVAD, and to a lesser extent DEVD, drastically inhibited ATP-dependent accumulation of extracellular but not intracellular IL-1 $\beta$ , pointing to a tight coupling between maturation and release. On the contrary, it is of interest that in mouse macrophages the proteolytic process and release appear to be uncoupled since a large amount of unprocessed IL-1 $\beta$  was detected in the supernatant of these cells challenged with ATP in the presence of YVAD (22). This observation might suggest that this ICE/caspase 1 blocker has a tissue-specific effect on IL-1 $\beta$  secretion, and thus it might have a more potent anti-inflammatory effect in the brain than in other tissues.

In conclusion, our study shows that in mouse microglia IL-1 $\beta$  secretion is a two-step process requiring first IL-1 $\beta$  gene transcription and pro-IL-1 $\beta$  accumulation, then activation of ICE/caspase 1 to promote maturation. The second stimulus consists of an unusual second messenger: a drop in the cytoplasmic K<sup>+</sup> concentration. Among various physiological agents, extracellular ATP via the P2X<sub>7</sub> receptor is the most likely candidate to the role of extracellular trigger for ICE/caspase 1 activation. The use of selective P2X<sub>7</sub> antagonists as anti-inflammatory drugs might be worth exploring.

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