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# Astrocyte-Derived ATP Induces Vesicle Shedding and IL-1 $\beta$ Release from Microglia<sup>1</sup>

Fabio Bianco,\*<sup>†</sup> Elena Pravettoni,\*<sup>†</sup> Alessio Colombo,\*<sup>†</sup> Ursula Schenk,\*<sup>†</sup> Thomas Möller,<sup>‡</sup> Michela Matteoli,\*<sup>†</sup> and Claudia Verderio<sup>2\*†</sup>

ATP has been indicated as a primary factor in microglial response to brain injury and inflammation. By acting on different purinergic receptors 2, ATP is known to induce chemotaxis and stimulate the release of several cytokines from these cells. The activation of purinergic receptors 2 in microglia can be triggered either by ATP deriving from dying cells, at sites of brain injury or by ATP released from astrocytes, in the absence of cell damage. By the use of a biochemical approach integrated with video microscopy experiments, we investigated the functional consequences triggered in microglia by ATP released from mechanically stimulated astrocytes, in mixed glial cocultures. Astrocyte-derived ATP induced in nearby microglia the formation and the shedding of membrane vesicles. Vesicle formation was inhibited by the ATP-degrading enzyme apyrase or by P2X<sub>7</sub>R antagonists. Isolation of shed vesicles, followed by IL-1 $\beta$  evaluation by a specific ELISA revealed the presence of the cytokine inside the vesicular organelles and its subsequent efflux into the extracellular medium. IL-1 $\beta$  efflux from shed vesicles was enhanced by ATP stimulation and inhibited by pretreatment with the P2X<sub>7</sub> antagonist oxidized ATP, thus indicating a crucial involvement of the pore-forming P2X<sub>7</sub>R in the release of the cytokine. Our data identify astrocyte-derived ATP as the endogenous factor responsible for microvesicle shedding in microglia and reveal the mechanisms by which astrocyte-derived ATP triggers IL-1 $\beta$  release from these cells. *The Journal of Immunology*, 2005, 174: 7268–7277.

Microglial cells play a major role in the inflammation processes which take place in the nervous tissue by secretion of specific cytokines. Among agents reported to promote cytokine release, ATP, acting at high concentrations on the ionotropic P2X<sub>7</sub> purinergic receptors, is one of the most powerful stimuli for the processing and release of IL-1 $\beta$ , the key initiator of acute inflammatory response (1). The physiological source of ATP needed to trigger cytokine release is still debated. Large amounts of the purine, accumulated extracellularly at the site of lesion, can directly activate P2X<sub>7</sub>-mediated cytokine release (2). In contrast, cytokine secretion at sites far away from damaged cells could proceed through the spreading of ATP-mediated calcium signals among astrocytes (3, 4). In a previous study we reported that ATP released by astrocytes during calcium wave propagation can activate P2X<sub>7</sub>Rs on microglial cells (3). Therefore, in principle, astrocyte-released ATP could be relevant for the control of cytokine secretion from microglia.

The signaling mechanism by which P2X<sub>7</sub> activation regulates IL-1 $\beta$  release in microglia is not completely defined yet, although it involves activation of caspase-1, which proteolytically cleaves the biological inactive precursor of the cytokine (pro-IL-1 $\beta$ ), gen-

erating the mature, active form (2). Caspase-1 activation is promoted both by calcium release from intracellular stores (5) and by K<sup>+</sup> efflux through P2X<sub>7</sub>Rs, which ultimately triggers phospholipase A<sub>2</sub> activation (6, 7). Release of IL-1 $\beta$  also requires the up-regulation of pro-IL-1 $\beta$  gene expression, which is triggered by cell exposure to bacterial LPS (8). However the molecular mechanism by which bioactive IL-1 $\beta$  is released is still debated. IL-1 $\beta$  lacks a conventional secretory sequence and is known not to be released through the classical endoplasmic reticulum (ER)<sup>3</sup>-Golgi pathway (9). Alternative pathways of secretion have been proposed in different cell types, including simple cell lysis (10), fusion of endolysosome-related vesicles (9), bleb formation, and shedding of membrane vesicles (11). The latter mechanism, which commonly occurs in hemopoietic and immune cells (12), represents a process whereby signal molecules are released via membrane-enclosed particles into the microenvironment. Based on the finding that IL-1 $\beta$  is detected inside shed vesicles shortly after P2X<sub>7</sub> activation and becomes detectable in the extracellular medium only at later time, it has been suggested that formation and shedding of membrane vesicles could represent a pathway for the release of the cytokine (11). More recently it has been suggested in murine macrophages that IL-1 $\beta$  secretion can be dissociated from P2X<sub>7</sub>-dependent formation of blebs at the plasma membrane, although a role of ATP-induced shed microvesicle in mediating IL-1 $\beta$  secretion has not been excluded (13). Because microglia has been indicated as the major source of IL-1 $\beta$  in the brain, it is of crucial importance to define the molecular mechanisms which mediate the cytokine release from these cells. Aims of the present study were to investigate 1) whether IL-1 $\beta$  is released from microglia similarly to monocytes, through the shedding of membrane vesicles, and 2) whether astrocyte-derived ATP can control the cytokine

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<sup>3</sup> Abbreviations used in this paper: ER, endoplasmic reticulum; GFAP, glial fibrillar acidic protein; LDH, lactate dehydrogenase; oATP, oxidized ATP; PS, phosphatidylserine; P2Rs, purinergic receptor 2; FLICA, fluorochrome inhibitor of caspases; SNAP-23, synaptosomal-associated protein of 23 kDa.

production from microglial cells. Our results indicate that ATP released from astrocytes modulates the cytokine release, which proceeds in microglia through the shedding of plasma membrane vesicles, containing packages of IL-1 $\beta$ . Our data also suggest a role for the pore-forming P2X<sub>7</sub>Rs in the processing and release of the cytokine from shed vesicles into the extracellular medium.

## Materials and Methods

### Primary cocultures of astrocytes and microglial cells

Primary mixed glial cultures from embryonic rat pups (embryonic days 18–19) were obtained as previously described (14). The dissociated cells were plated onto glass coverslips at a density of  $0.5 \times 10^6$  cells/ml, and grown in MEM (Invitrogen Life Technologies) supplemented with 20% FCS (Euroclone) and 100 IU/ml penicillin, 10 mg/ml streptomycin, and 5.5 g/L glucose (glial medium). Purified microglial cultures were harvested by shaking 3-wk-old mixed glial cultures. Detached microglia was seeded on glass coverslips and cultured in the same medium. To obtain a pure astrocyte monolayer, mixed cultures were maintained in a serum-free medium for 2–3 days, and microglia cells were removed by vigorously shaking the cultures. Microglial cells present in mixed glial cultures were distinguished from astrocytes by morphological criteria and retrospective immunostaining for CSF-1R and glial fibrillar acidic protein (GFAP), respectively. The absence of microglia and astrocyte contamination in primary astrocytes and purified microglia cultures was assessed by Western blotting for the microglial marker CSF-1R (Molecular Probes) and the astrocytic marker GFAP (Sigma-Aldrich).

### Microglial N9 cell line

The N9 murine microglial cell line was generated by infecting embryonic brain cultures with the 3RV retrovirus carrying an activated *v-myc* oncogene (15). N9 cells were maintained in IMDM (Invitrogen Life Technologies) supplemented with 100 IU/ml penicillin, 10 mg/ml streptomycin, 2 mM L-glutamine, and 50 nM 2-ME (Invitrogen Life Technologies) at 37°C and 5% CO<sub>2</sub>. To obtain cocultures of N9 cells and astrocytes, N9 cells were plated at a density of  $1.2 \times 10^5$  cells/ml on top of astrocytes previously cultured for 7–10 days on glass coverslips or 60-mm petri dishes.

N9 cells stably expressing GFP were obtained as described in Ref. 16.

### Western blot analysis of IL-1 $\beta$ processing and release

Approximately 4,500,000 cells were primed for 6 h with 100 ng/ml LPS and stimulated with 1 mM ATP in 1 ml of a Krebs-Ringer solution (KRH) for 30 min (125 mM NaCl, 5 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 6 mM D-glucose, and 25 mM HEPES/NaOH, pH 7.4). The conditioned saline was then collected and concentrated by  $2,340 \times g$  centrifugation at 4°C on centrifugal filters with a 10-kDa cut-off (Millipore), for ~6 h to a final volume of 150  $\mu$ l, run on a 12% polyacrylamide gel and blotted onto nitrocellulose filters (Millipore). IL-1 $\beta$  was detected with goat anti-mouse Abs (R&D Systems) followed by secondary HRP-conjugated anti-goat Abs (Jackson ImmunoResearch Laboratories) and revealed using an ECL system (Supersignal from Pierce) following the manufacturer's protocol. Anti-IL-1 $\beta$  Ab recognizes both the 36-kDa precursor (pro-IL-1 $\beta$ ) and the 17-kDa active form of the cytokine.

### ELISA analysis of IL-1 $\beta$

A mouse IL-1 $\beta$  ELISA kit, which does not discriminate between pro- and mature cytokine forms (Pierce Endogen), was used to quantify the presence of IL-1 $\beta$  in the supernatant of N9 cells. Approximately 1,000,000 cells were preactivated with 100 ng/ml LPS for 6 h and stimulated with 1 mM ATP for different time periods (5, 10, 20, and 30 min) in 1 ml of culture medium. Conditioned media were then collected, and the assay was completed following the manufacturer's protocol, adding, in each well, 50  $\mu$ l of collected medium to 50  $\mu$ l of biotinylated Ab. Release of IL-1 $\beta$  from shed vesicles was directly evaluated by assaying the medium (50  $\mu$ l) in which vesicles were maintained for different times (10, 20, and 30 min) at 37°C after isolation. IL-1 $\beta$  content inside shed vesicle was determined after detergent permeabilization with 2% Triton X-100. Sample absorbance was measured with a spectrophotometric system (1420 Multilabel Counter Victor 2; Wallac) at 450 nm at 10 Hz. The actual IL-1 $\beta$  concentration was estimated on the basis of a standard curve at known concentration of IL-1 $\beta$ , both in the presence and in the absence of 2% Triton X-100.

### Annexin V<sup>FITC</sup> staining and microscope counting

N9 cells were either directly plated at a density of  $1.2 \times 10^5$  cells/ml on glass coverslips or plated on top of astrocytes previously cultured for 7–10

days. Sixteen hours after plating, coverslips were either maintained in static condition or exposed to mechanical stimulation for 1 min in KRH with or without apyrase (30 U/ml). Mechanical stimulation was conducted by shaking the petri dishes onto an orbital shaker at a rate of 160 per min (17) (Orbital Shaker SO3; Stuart Scientific). Stimulations were repeated two to three times, with a 5-min pause in between stimuli. Alternatively, the mechanical stimulation was conducted by the gentle mechanical contact of a patch pipette with the surface of a single astrocyte either in primary astrocyte-microglia cocultures or in astrocyte-N9 cocultures. Mechanically stimulated cultures were incubated with Annexin V<sup>FITC</sup> (4.2  $\mu$ g/ml) for 3 min and the number of annexin V-positive N9 cells in living cultures was determined by counting positive cells in at least 50 microscope fields (Axiovert 100; Zeiss). In a set of experiments, cultures were fixed after Annexin V<sup>FITC</sup> staining with 4% paraformaldehyde in 0.12 M phosphate buffer containing 0.12 M sucrose, and images were acquired using a Bio-Rad MCR-1024 confocal microscope equipped with LaserSharp 3.2 software (Bio-Rad Hercules).

### Vesicle isolation

Approximately 4,500,000 cells, primed with LPS, were exposed to 1–3 mM ATP for 10 min under gentle rotation in 1 ml of KRH. The supernatant, containing shed vesicles, was withdrawn and incubated for 10 min at 4°C under gentle periodic rotation with streptavidin beads (Sigma-Aldrich), precoated with biotinylated annexin V (Sigma-Aldrich). Shed vesicles bound to annexin-coated beads were then separated from the supernatant by gravity sedimentation at 4°C, treated with a reducing sample buffer and run on a 12% polyacrylamide gel. Alternatively, vesicles bound to beads were permeabilized with Triton X-100 (2%) in 100  $\mu$ l of culture medium and probed for IL-1 $\beta$  content by ELISA (Pierce Endogen). Caspase-1 activity in isolated vesicles was quantified by a carboxyfluorescein-fluorochrome inhibitor of caspases (FLICA) assay kit (B-Bridge International), based on the use of the caspase-1 fluorescent inhibitor FAM-YVAD-FMK. The FAM-YVAD-FMK FLICA probe enters the cells and covalently binds to a reactive cysteine residue, thereby inhibiting further enzymatic activity. The assay was conducted following the manufacturer's protocol.

### Analysis of vesicle shedding

To visualize shed vesicles present in the supernatant of ATP-stimulated cells, ~1,000,000 N9 cells were labeled with the fluorescent styryl dye FM1-43 (2  $\mu$ M) for 2 min, extensively washed and exposed for 20 min to 1 mM ATP with or without 500  $\mu$ M EDTA or 100  $\mu$ M oxidized ATP (oATP) in 1 ml of KRH. Supernatant was then deposited on glass coverslips precoated with poly-L-lysine (Sigma-Aldrich). Images of sedimented vesicles were acquired 20 min after supernatant addition and analyzed using Metamorph Imaging Series 6.1 software (Universal Imaging). Alternatively, to quantify the amount of shed vesicles present in supernatants under different experimental conditions, total green fluorescence of collected supernatants was assayed at 485/535 nm and 10 Hz with a spectrophotometric system (1420 Multilabel Counter Victor 2; Wallac). A similar spectrophotometric analysis was performed on supernatants containing GFP vesicles, shed from N9 cells expressing cytosolic GFP.

### Analysis of vesicle integrity

To investigate whether shed vesicles disrupt with time, thus allowing the release of pro- and active-IL-1 $\beta$  into the extracellular medium, we monitored the presence of GFP in vesicles shed from GFP-mutant N9 cells, and in vesicle-depleted medium 10, 20, and 30 min after vesicle isolation. GFP is a 40-kDa cytosolic protein that cannot be released extracellularly through the P2X<sub>7</sub> pore, given that the channel-pore is permeable to molecules up to ~1 kDa. Total green fluorescence in vesicle and vesicle-free fractions was assayed at 485/535 nm as described above.

### Video microscopy

Cells were mounted on an inox cell chamber (Elettrofor) and observed at 37°C, 5% CO<sub>2</sub>, and 55–60% relative humidity using an inverted Zeiss Axiovert 200M microscope equipped with a chamber Incubator S (Zeiss) controlled by a CTI Controller 3700 (Zeiss). Images were acquired with a digital CCD camera system Micromax 512 BFT (Princeton Instruments) and analyzed using Metamorph Imaging Series 6.1 software. Cells were considered as blebbing when at least three blebs per cell were clearly detectable.

### Cytotoxicity assay

Aliquots of culture medium were collected at different time points (10, 20, and 30 min) for the evaluation of lactate dehydrogenase (LDH) activity by

a cytotoxicity assay (Promega). LDH levels in culture supernatant were measured via a coupled enzymatic assay, which resulted in the formation of a colored product, the absorbance of which was recorded at 490 nm and 10 Hz (1420 Multilabel Counter Victor 2; Wallac). Caspase-3 activity in N9 cells exposed to 1 mM ATP for different time periods was evaluated using a carboxyfluorescein FLICA assay (B-Bridge International). Briefly, cells were detached from culture substrate using 0.05% trypsin, 0.02% EDTA in PBS (Euroclone) and then exposed to ATP in KRH solution. Sample absorbance was measured with a spectrophotometric system (1420 Multilabel Counter Victor 2; Wallac) at 450 nm at 10 Hz.

### Chemicals

Rabbit Abs against synaptosomal-associated protein of 23 kDa (SNAP-23) were kindly provided by Dr. T. Gally (Institut National de la Santé et de la Recherche Médicale, Paris, France). Rabbit Abs against P2X<sub>7</sub> was obtained from Alomone Labs. Anti-GFAP Abs, Annexin V<sup>FITC</sup>, biotinylated annexin V, oATP, apyrase, LPS, ATP were obtained from Sigma-Aldrich. Abs anti-CSF-1R were purchased from Santa Cruz Biotechnology. Rabbit Abs against ribophorin were kindly provided by Dr. G. Kreibich (New York University, New York, NY). FM1-43 was from Molecular Probes. The polyclonal Abs against human cathepsin D were generously provided by Dr. C. Isidoro (University of Novara, Novara, Italy). Rabbit Abs against LAMP-1 (lgp120) were a kind gift of Dr. I. Mellman (Yale University, New Haven, CT). Rabbit Abs against rab-7 were a kind gift of Dr. R. Jahn (Max Planck Institute, Göttingen, Germany).

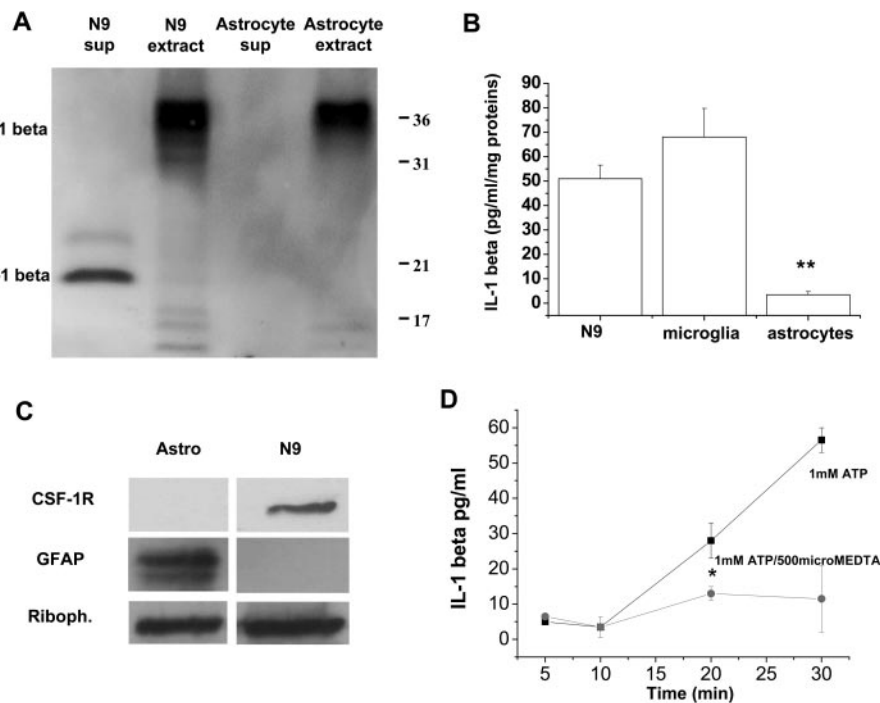
### Data analysis

The data are presented as means  $\pm$  SE. Statistical significance was evaluated by the Student's test or one-way ANOVA. Differences were considered significant if  $p < 0.05$  and are indicated by an asterisk in all figures, whereas those at  $p < 0.01$  are indicated by double asterisks.

## Results

### Microglia, but not hippocampal astrocytes, release IL-1 $\beta$ upon ATP exposure

Several evidences have been reported demonstrating that large amounts of exogenous ATP induce IL-1 $\beta$  processing and release from microglia, the cell type which serves as a major source of the cytokine in the CNS upon injury or inflammation (18). Less clear is the scenario in astrocytes, given that most of the evidence for IL-1 $\beta$  release from these cells derives from studies using mixed primary cultures, containing both astrocytes and microglia (19). To verify, in our experimental model, the relative contribution of microglia and astrocytes to IL-1 $\beta$  production, either N9 microglia cells or cultures of pure hippocampal astrocytes were stimulated for 30 min with 1 mM ATP after 6 h of LPS priming. Although both cell types express P2X<sub>7</sub>Rs and respond with intracellular calcium elevations to 100  $\mu$ M BzATP (20, 21), an agonist rather selective for this receptor subtype, the mature form of IL-1 $\beta$  (17 kDa) was detected, by Western blot analysis, in the supernatant of N9 cells but not in the supernatant of primary astrocytes (Fig. 1A). In contrast, the immature form of the cytokine (36 kDa) was clearly detectable in both astrocyte and N9 homogenates (Fig. 1A). In the absence of ATP treatment, the mature form of IL-1 $\beta$  fails to be detected in N9 supernatant (data not shown). These data indicated that, at least in our experimental model, hippocampal astrocytes are not able to process and release IL-1 $\beta$  upon purinergic receptor 2 (P2R) activation. The ELISA analysis of IL-1 $\beta$



**FIGURE 1.** ATP induces IL-1 $\beta$  release from microglial cells but not from hippocampal astrocytes. *A*, Western blot analysis for IL-1 $\beta$  on cell lysates and supernatants of N9 cells and primary hippocampal astrocytes, primed 6 h with 100 ng/ml LPS and incubated with 1 mM ATP for 30 min. One representative experiment of three is shown. *B*, IL-1 $\beta$  detection by ELISA in the supernatants collected from N9 cells, hippocampal microglia, and hippocampal astrocytes primed 6 h with 100 ng/ml LPS and incubated with 1 mM ATP for 30 min. Values are presented as mean  $\pm$  SE picograms per milliliter obtained from three independent experiments and are normalized to protein concentration of cell extracts (51.0  $\pm$  5.56 pg/ml/mg proteins in N9 supernatants; 68.0  $\pm$  11.79 pg/ml/mg proteins in medium collected from purified microglia; 3.5  $\pm$  1.35 pg/ml/mg proteins in medium collected from purified astrocytes, one-way ANOVA, post hoc Tukey's method,  $p < 0.01$ ). *C*, Representative Western blotting of primary astrocytes for the microglial marker CSF-1R and the astrocytic marker GFAP, revealing the absence of microglia contamination in astrocytic cultures. The ER marker ribophorin was used as loading control. *D*, Kinetics of IL-1 $\beta$  secretion, as measured by ELISA. LPS-activated N9 cells were exposed to 1 mM ATP for different times in normal cell culture medium or in medium supplemented with 500  $\mu$ M EDTA. Chelation of calcium ions in the extracellular medium almost completely inhibits the release of the cytokine. Values are presented as mean  $\pm$  SE obtained from three independent experiments. Statistical significance was evaluated by Student's test ( $p = 0.015$  at 20 min;  $p = 0.005$  at 30 min,  $n = 3$ ).



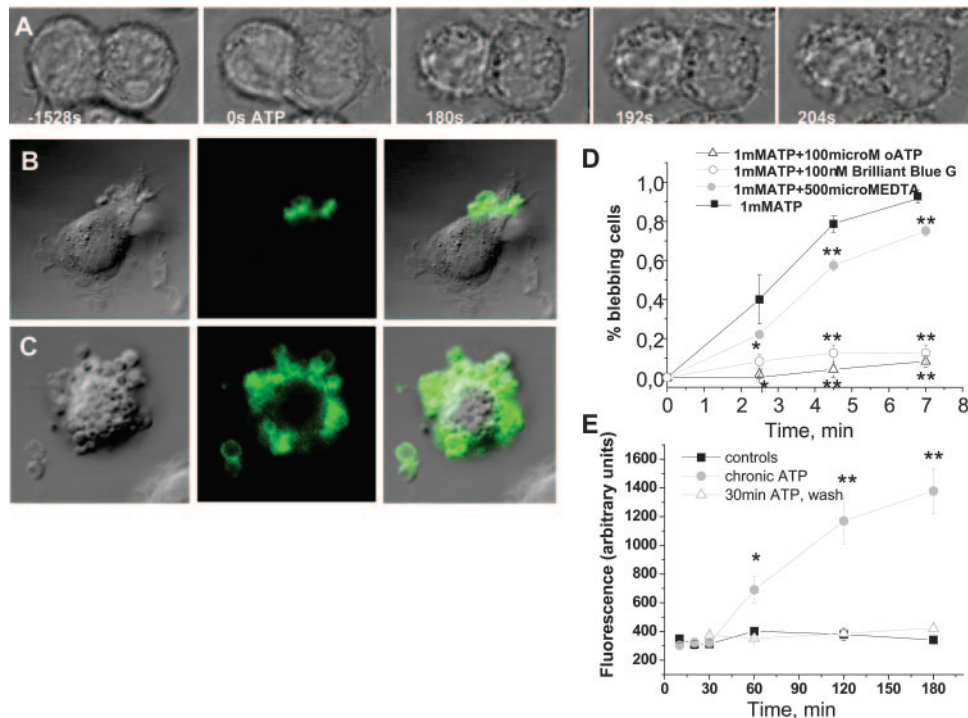
levels in the cell supernatants of N9 cells, primary purified microglia and primary hippocampal astrocytes further confirmed that ATP stimulation induces IL-1 $\beta$  release essentially from microglia (Fig. 1*B*). In the absence of ATP stimulation, IL-1 $\beta$  levels were below the assay sensitivity. Western blot analysis for CSF-1R and GFAP, specific markers of microglia and astrocytes (3) ruled out the possible contamination of astrocytic cultures with primary microglia (Fig. 1*C*). The kinetic analysis of IL-1 $\beta$  release from microglial N9 cells indicated that significant levels of the cytokine are present extracellularly 15–20 min after ATP stimulation. In line with previous results, removal of calcium ions from the extracellular medium by addition of 500  $\mu$ M EDTA strongly prevented the release of the cytokine, thus suggesting a calcium-dependent mechanism of secretion (22) (Fig. 1*D*).

#### Exposure to exogenous ATP induces vesicle formation in microglial cells

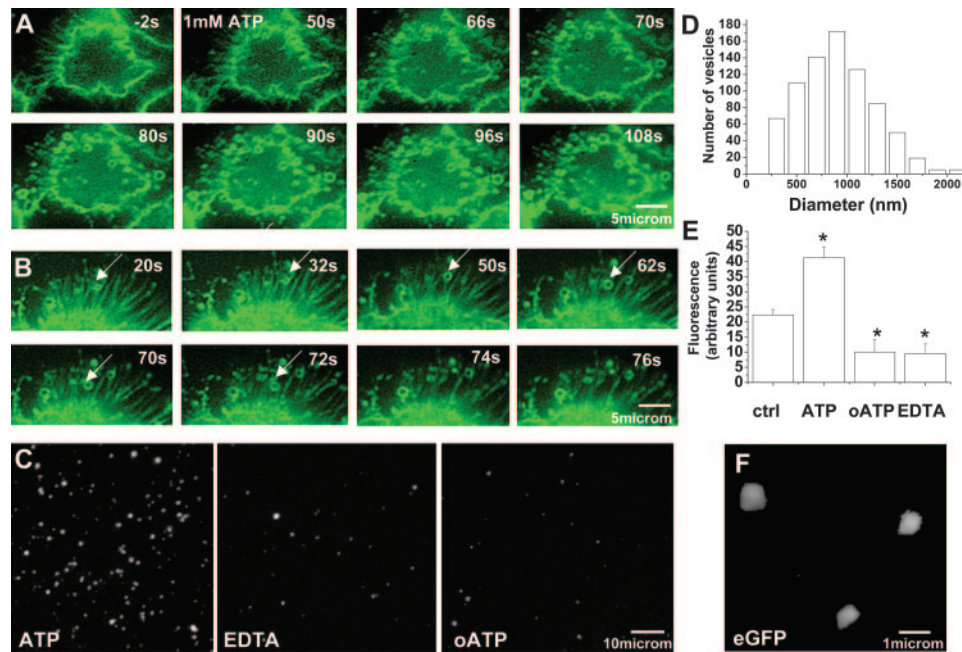
To investigate whether ATP stimulation induces vesicle formation in microglia, video microscopy experiments were conducted upon 1 mM ATP treatment both in primary hippocampal microglia and in N9 cells. Bright field time-lapse microscopy revealed the formation of plasma membrane structures (blebs) continuously extruding and retracting from the cell membrane (Fig. 2*A*),  $\sim$ 180 s after 1 mM ATP addition. As already described for platelets and monocytes (11, 23), in microglia ATP-induced blebs have phosphatidylserine (PS) exposed on the outer leaflet of the plasma membrane and bind annexin V, a high affinity ligand for the phospholipid. Annexin V-positive vesicle formation was confined to

discrete regions of the cell surface (Fig. 2*B*) or more evenly distributed to the entire surface of the cells (see an extreme case in Fig. 2*C*). Vesicle formation proceeded even in the absence of extracellular calcium ions, although with a latency of  $\sim$ 60 s as compared with controls (Fig. 2*D*); calcium chelation by EDTA did not prevent annexin V binding to the forming vesicles (number of annexin-positive cells per field increases by  $4.44 \pm 0.18$ -fold upon ATP treatment, by  $2.9 \pm 0.086$  upon ATP treatment in the presence of EDTA). Pretreatment of N9 cells with 100  $\mu$ M oATP or 100 nM Brilliant Blue G, two antagonists rather selective for the P2X<sub>7</sub>Rs (24, 25), quite completely inhibited vesicle formation, thus suggesting the requirement of P2X<sub>7</sub>R activation for ATP-induced bleb formation (Fig. 2*D*). ATP-induced vesicle formation also occurred in microglial cells not primed with LPS (data not shown).

After a brief ATP exposure (4–5 min) both N9 and primary microglia were able to recover the cell shape, indicating that bleb formation is not necessarily linked to an apoptotic fate of the cell. Accordingly, a negligible increase of LDH concentration was detected in medium collected from N9 cells exposed to ATP up to 20 min (increase of LDH concentration:  $4.83 \pm 0.89\%$ ). Furthermore, in line with previous results indicating that blebbing induced by ATP does not require activity of the apoptotic marker caspase-3 (13), no significant activation of caspase-3 was observed in N9 cells exposed to ATP up to 30 min. Caspase-3 activity became detectable in N9 cells only after 60 min of chronic ATP treatment (Fig. 2*E*), thus excluding that ATP-induced bleb formation, which



**FIGURE 2.** ATP causes formation of membrane blebs. *A*, Series of bright field images of two primary hippocampal microglial cells taken at different times before and after exposure to 1 mM ATP. Images reveal that ATP induces the formation of vesicular structures continuously extruding and retracting from the cell membrane. *B* and *C*, Differential interference contrast and fluorescence images (490 nm) of N9 cells incubated with FITC-conjugated annexin V after a 5-min treatment with 1 mM ATP. Formation of annexin V-positive blebs can be confined to a discrete region of the plasma membrane, suggesting a focalized process (*B*) or localized to the entire cell surface (an extreme case is shown in *C*). *D*, The graph shows the percentage of N9 cells displaying blebs at different time points after continuous ATP stimulation, based on the analysis of time lapse images. Pretreatment of N9 cells with P2X<sub>7</sub> antagonists oATP and Brilliant Blue G significantly inhibits bleb formation. Removal of calcium ions from the extracellular medium slows down bleb appearance by  $\sim$ 2 min. (values are compared with ATP-stimulated cells. Statistical significance was evaluated by one-way ANOVA, post hoc Tukey's method,  $n = 3$ ). *E*, Caspase-3 activity assay on N9 cells exposed to 1 mM ATP for different time periods. A significant level of caspase-3 activity is detected after 60 min of chronic exposure to ATP. No significant increase in the enzyme activity was observed in cells exposed to ATP for up to 30 min (statistical significance was evaluated by one-way ANOVA, post hoc Dunnett's method,  $n = 3$ ).



**FIGURE 3.** Vesicle shedding from microglia exposed to exogenous ATP. *A* and *B*, Fluorescence images of two different fields of N9 cells labeled with FM1-43 during ATP exposure. Frames were taken at the indicated times before and after ATP addition. Note that ATP treatment induces formation of FM1-43-labeled vesicles which appear loosely associated with cell margins. Formation and budding of vesicles from the plasma membrane illustrated in *B* can be also seen in the supplementary movie. The arrow points to a vesicle which moves along microglial filopodia and eventually detaches from the cell membrane. *C*, Fluorescence images of FM1-43-labeled vesicles collected from supernatant of N9 cells exposed to ATP and then sedimented on glass coverslips. Note a clear reduction in the number of vesicles shed from N9 cells exposed to ATP either in the absence of extracellular calcium ions or after culture treatment with 100  $\mu$ M oATP. *D*, Frequency histogram indicating the size distribution of shed vesicles. *E*, The histogram shows the spectrophotometric analysis of supernatants collected from N9 cells containing FM1-43-labeled vesicles. Note a significant reduction in the fluorescence of supernatants from cells stimulated either in the absence of extracellular calcium ions or after treatment with oATP (fluorescence arbitrary units:  $22.5 \pm 2.5$ , control;  $42.99 \pm 2.13$ , ATP;  $4.56 \pm 0.56$ , oATP;  $6.36 \pm 1.16$ , EDTA,  $n = 3$ ,  $p = 0.016$ , one-way ANOVA, post hoc Dunnett's method). *F*, Fluorescent image of vesicles shed from GFP-N9 microglia cells.

takes place within 4–5 min from ATP addition, is necessarily linked to apoptotic cell death.

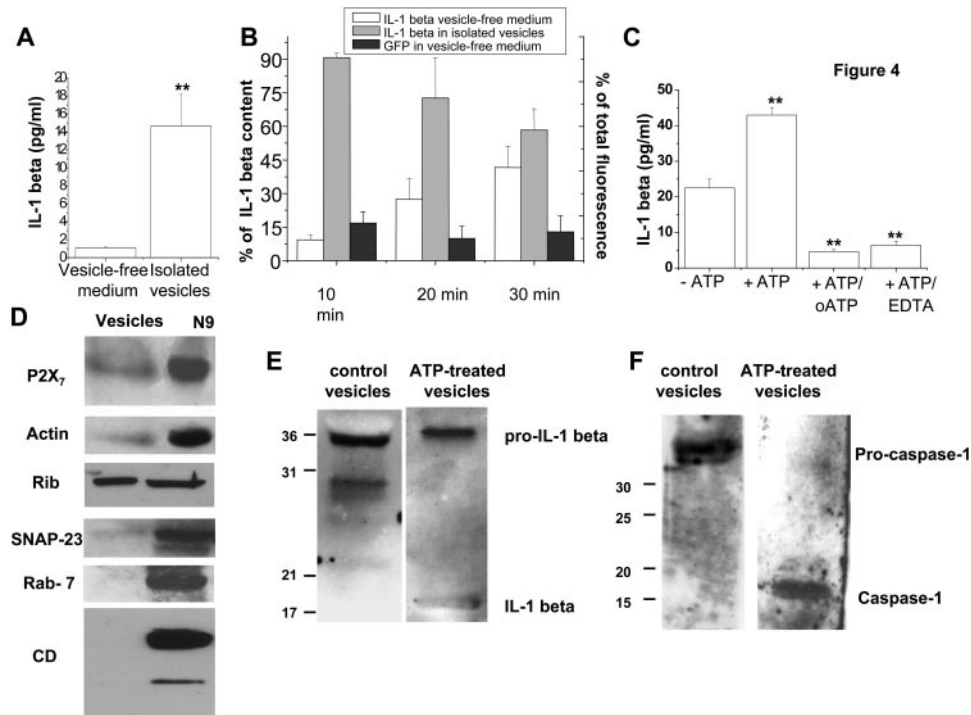
To better follow the dynamics of bleb formation in ATP-stimulated microglia we conducted fluorescence video microscopy on cells incubated with the fluorescent styryl dye FM1-43, which labels lipid bilayers. By this approach, we clearly observed not only the formation but also the shedding from the plasma membrane of vesicular structures of variable size (Fig. 3, *A* and *B*) (see also supplemental movie).<sup>4</sup> To visualize vesicles shed into the extracellular medium, the supernatant collected from FM1-43-labeled cells exposed to ATP was deposited on glass coverslips. As shown in Fig. 3*C* collected supernatants contained a heterogeneous population of FM1-43-labeled vesicles (mean diameter value 980 nm,  $n = 780$ ) (Fig. 3*D*). Pretreatment with 100  $\mu$ M oATP or removal of calcium ions from the extracellular saline significantly reduced the number of shed vesicles in the supernatants (Fig. 3*C*). Accordingly the amount of fluorescence in N9 collected supernatants, due to the presence of FM1-43-labeled vesicles, was significantly reduced when cells were exposed to ATP in the absence of calcium ions and when cells were pretreated with oATP (Fig. 3*E*). Similar experiments were conducted on a clone of N9 cells, which stably express high levels of GFP in the cytosol. GFP-expressing N9 cells shed GFP-vesicles upon ATP stimulation. An example of GFP-vesicles are shown in Fig. 3*F*. Quantitative analysis of total green fluorescence of supernatants containing GFP-vesicles confirmed that vesicle shedding is markedly inhibited by calcium chelation (fluorescence intensity, arbitrary units:  $18.83 \pm$

$0.4$  controls,  $105.83 \pm 2.35$  ATP,  $3.67 \pm 0.2$  ATP + EDTA,  $n = 3$ ,  $p = 0.004$ , one-way ANOVA, post hoc Dunnett's method) differently from ATP-induced bleb formation which appeared to be only slightly delayed by EDTA (Fig. 2*D*).

#### Shed vesicles contain IL-1 $\beta$

To verify whether vesicle formation could mediate IL-1 $\beta$  release from microglia, shed vesicles were isolated from the supernatant of LPS-primed N9 cells, 8–10 min after ATP stimulation, when low levels of IL-1 $\beta$  were detectable in the extracellular medium (Fig. 1*D*). Shed vesicles were isolated using annexin V-coated beads, permeabilized with 2% Triton X-100 to release vesicle content and assayed for the presence of IL-1 $\beta$ . High levels of IL-1 $\beta$  were detected in shed vesicles ( $14.66 \pm 3.5$  pg/ml) while minimum amounts of the cytokine were observed in the vesicle-depleted medium ( $1.08 \pm 0.14$  pg/ml,  $n = 3$ ,  $p = 0.008$ , Fig. 4*A*). The amount of IL-1 $\beta$  measured in Triton X-100-treated supernatant of N9 cells (i.e., total extracellular IL-1 $\beta$ ) at 10 min was  $\sim 27\%$  of the total cellular content, in agreement with data from Sanz and Di Virgilio (2) who reported a releasable fraction of  $\sim 25\%$ . To investigate whether the cytokine could be released from shed vesicles into the extracellular medium, isolated vesicles were maintained in KRH at 37°C for different time periods. The evaluation of IL-1 $\beta$  content of both vesicular and vesicle-free supernatant fractions indicated a progressive flow of the cytokine from shed vesicles into the extracellular medium (IL-1 $\beta$  in vesicle-free supernatant: 10 min =  $1.25 \pm 0.35$  pg/ml corresponding to  $9.36 \pm 1.97\%$  of total IL-1 $\beta$  present in the extracellular medium; 20 min =  $11.5 \pm 3.5$  pg/ml corresponding to  $27.37 \pm 9.3\%$  of total IL-1 $\beta$  30 min =  $22.5 \pm$

<sup>4</sup> The online version of this article contains supplemental material.



**FIGURE 4.** P2X<sub>7</sub> mediated IL-1 $\beta$  processing and release from shed vesicles. *A*, IL-1 $\beta$  detection by ELISA in the vesicle fraction and in the vesicle-free medium of N9 cells after a 10 min exposure to 1 mM ATP. *B*, Histogram showing time course of 1) IL-1 $\beta$  content of isolated vesicles (gray bars) and medium in which vesicles are contained (white bars), and 2) GFP content in vesicle-free medium (dark gray bars). Values represent mean  $\pm$  SE percentage of total GFP, i.e., vesicular plus extracellular GFP, obtained from three independent experiments (10 min =  $16.8 \pm 5.00\%$ ; 20 min =  $10.00 \pm 5.3\%$ ; 30 min =  $12.8 \pm 7.2\%$ ). *C*, Quantitative analysis of IL-1 $\beta$  content in the extracellular medium after a 30-min incubation with shed vesicles. Note that ATP stimulation enhances IL-1 $\beta$  release from shed vesicles while oATP pretreatment or extracellular calcium chelation reduces IL-1 $\beta$  release below control condition. *D*, Western blot analysis performed on extracts from shed vesicles and N9 cells extracts, for P2X<sub>7</sub>Rs, actin, the ER marker ribophorin (Rib), the plasma membrane protein SNAP-23 and the endolysosomal markers rab-7 and cathepsin D (CD). Isolated vesicles are not immunoreactive for the endolysosomal markers. *E*, Western blot analysis of shed vesicles for IL-1 $\beta$ , revealing the presence of procytokine inside vesicles lysed immediately after isolation and of both pro- and active-IL-1 $\beta$  in vesicles incubated for 30 min at 37°C with 1 mM ATP after isolation. *F*, Representative Western blot showing procaspase-1 in shed vesicles lysed immediately after isolation and the presence of the mature form of the enzyme in vesicles exposed for 30 min to 1 mM ATP after isolation.

2.5 pg/ml corresponding to  $41.6 \pm 9.5\%$  of total IL-1 $\beta$ ,  $n = 3$ , Fig. 4B). Cytokine release from shed vesicles was enhanced when vesicles were stimulated with 1 mM ATP (IL-1 $\beta$  in vesicle-free medium: control =  $22.5 \pm 2.5$  pg/ml, ATP =  $42.99 \pm 2.1$  pg/ml) while it was significantly inhibited when vesicles were pretreated with 100  $\mu$ M oATP ( $4.56 \pm 0.56$  pg/ml) or maintained in a calcium-free medium ( $6.36 \pm 1.16$  pg/ml), thus indicating the involvement of the pore-forming P2X<sub>7</sub>R in the release of the cytokine ( $n = 3$ ,  $p = 0.016$ , one-way ANOVA, post hoc Dunnett's method, Fig. 4C). Interestingly, IL-1 $\beta$  concentration in the extracellular medium was reduced even below control level after vesicle exposure to oATP. This may suggest a partial activation of P2X<sub>7</sub>R on shed vesicles, due to the previous ATP treatment needed to induce vesicle shedding. The gradual increase of IL-1 $\beta$  in the extracellular medium does not appear to result from progressive vesicle disruption, as indicated by the time course analysis of fluorescence in GFP-vesicle and GFP-vesicle-free medium, collected from N9 cells stably expressing cytosolic GFP (Fig. 4B). If GFP-vesicles broke with time, GFP would gradually flow into the vesicle-free supernatant. A similar percentage of GFP fluorescence was instead detected in vesicle-free fractions at all analyzed time points ( $16.8 \pm 5.0$ , 10 min;  $10 \pm 5.3$ , 20 min;  $12.8 \pm 7.22$ , 30 min; Fig. 4B). Accordingly, no significant reduction of GFP fluorescence was detected at different time points in isolated vesicles ( $65.5 \pm 4.1$ , 10 min;  $67.5 \pm 6.5$ , 20 min;  $62 \pm 5.16$ , 30 min). Western blot analysis revealed the presence of pro-IL-1 $\beta$  (Fig.

4E), procaspase-1 (Fig. 4F), and P2X<sub>7</sub>Rs (Fig. 4D) in vesicle fractions, solubilized immediately after isolation. Isolated vesicles were also stained for the ER marker ribophorin, for the plasma membrane protein SNAP-23 and for actin (Fig. 4D), in line with previous reports indicating that microvesicles and apoptotic blebs could carry a number of ER-derived proteins (26) and could contain cytoskeletal elements. Isolated vesicles were not immunoreactive for the endosomal (45 kDa) and lysosomal (31 kDa) forms of cathepsin D (Fig. 4D), for the endolysosomal markers rab-7 (Fig. 4D) and LAMP-1 (data not shown). Lack of cathepsin D and rab-7 staining in isolated vesicles but not in N9 homogenate, aside from indicating that isolated vesicles do not derive from lysosomal organelles, ruled out the possible contamination of isolated vesicles with apoptotic cell fragments (Fig. 4D). Interestingly, after 30 min of in vitro exposure to ATP, a processed band of both IL-1 $\beta$  and caspase-1 appeared in isolated vesicles (Fig. 4, E and F), suggesting that both precursors can be locally activated upon ATP stimulation (active-/pro-IL-1 $\beta$  ratio: 0.39 after 30 min of stimulation with 1 mM ATP,  $n = 3$ ). In line with this finding, quantitative analysis of active caspase-1 by FLICA assay showed an increase in the number of active caspase-1 enzymes upon ATP stimulation in isolated vesicles as compared with vesicles incubated in vitro in the presence of oATP, after isolation (fluorescence arbitrary units:  $601.85 \pm 105.95$ , ATP;  $147.1 \pm 76.0$ , ATP + oATP,  $p = 0.01228$ ,  $n = 2$ ).



Altogether, our results indicate that shed vesicles, isolated from ATP-stimulated microglia cells, contain pro-IL-1 $\beta$ , which is converted in the active cytokine upon ATP exposure and is progressively released into the external medium via a calcium and P2X<sub>7</sub>-dependent mechanism.

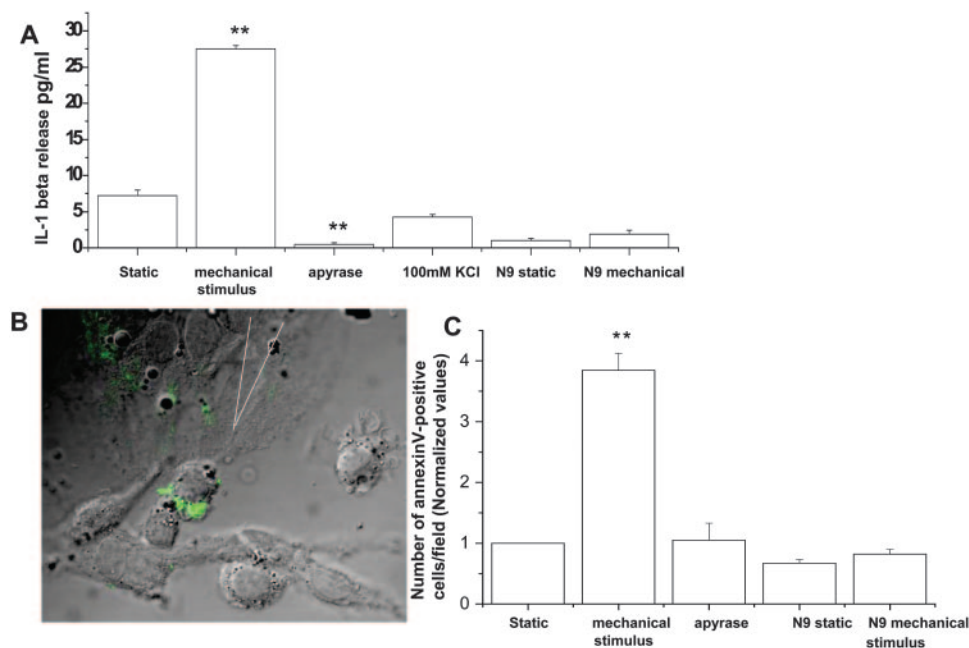
#### *Astrocyte-derived ATP stimulates IL-1 $\beta$ release from microglial cells*

To investigate whether ATP derived from astrocytes is sufficient to induce IL-1 $\beta$  release from adjacent microglial cells, we evaluated the presence of the cytokine in the supernatant of astrocyte-N9 cocultures. It has been previously shown that release of ATP from astrocytes is enhanced by mechanical stimulation (17, 27) and that astrocyte-derived ATP may activate P2Rs present in adjacent microglial cells (3, 4). To evaluate whether ATP release, induced by mechanical stimulation of astrocytes, triggers IL-1 $\beta$  secretion from adjacent microglia cells, astrocyte-N9 cocultures were mechanically stimulated on an orbital shaker, as previously described (17). ELISA analysis of collected media indicated a 3- to 4-fold increase of IL-1 $\beta$  concentration in the supernatant of mechanically stimulated cocultures ( $27.5 \pm 0.5$  pg/ml), as compared with supernatants from control cultures maintained under static conditions ( $7.25 \pm 0.75$  pg/ml) (Fig. 5A). Interestingly, IL-1 $\beta$  decreased even below control levels ( $0.5 \pm 0.05$  pg/ml) when mechanical stimulation was performed in the presence of the ATP-degrading enzyme apyrase (30 U/ml), which is known to significantly prevent the ATP-mediated astrocyte-to-microglia calcium signaling (3). IL-1 $\beta$  release from primary astrocyte-microglia cocultures was also markedly reduced when mechanical stimulation was performed in high extracellular concentration of K<sup>+</sup> (IL-1 $\beta$ ,  $27.5 \pm 0.5$  pg/ml, control medium;  $4.5 \pm 0.375$  pg/ml, high K<sup>+</sup> medium), thereby indicating that inhibition of P2X<sub>7</sub>-induced K<sup>+</sup> efflux (28) by as-

trocyte-derived ATP does repress the paracrine activation of caspase-1 and of IL-1 $\beta$  release from microglia. IL-1 $\beta$  concentration in the supernatant of N9 cultures in the absence of astrocytes was significantly lower as compared with cocultures ( $1.00 \pm 0.3$  pg/ml) and the mechanical stimulation of pure N9 cultures only slightly increased IL-1 $\beta$  concentration in the supernatant ( $1.92 \pm 0.5$  pg/ml) (Fig. 5A). These data suggested that astrocyte-derived ATP, released either spontaneously or upon mechanical stimulation, induces IL-1 $\beta$  secretion from N9 cells (Fig. 5A). An almost negligible contribution to cytokine release seems to be due to ATP derived from microglia (Fig. 5A).

#### *Astrocyte-derived ATP induces bleb formation in microglial cells*

To evaluate whether ATP released from astrocytes could be sufficient to induce vesicle formation in microglial cells, bright field video microscopy experiments were conducted on cocultures of astrocytes and either primary microglia or N9 cells. Gentle mechanical contact between a patch pipette and the surface of a single astrocyte induced in adjacent microglia the formation of vesicular structures, which bound Annexin V<sup>FITC</sup> (Fig. 5B). The quantitative analysis of annexin V-positive cells was conducted in astrocyte-N9 cocultures, mechanically stimulated on an orbital shaker. Fig. 5C showed a 3-fold increase in the number of annexin-positive N9 cells upon mechanical stimulation. Binding of annexin V was not significantly enhanced in pure N9 cultures, upon mechanical stimulus, thus excluding that the mechanical stress per se or ATP secretion from microglial cells play a relevant role in vesicle formation (number of annexin V-positive cells per field, normalized to static coculture condition:  $3.84 \pm 0.28$  cocultures, mechanical stimulation;  $1.05 \pm 0.28$ , cocultures, mechanical stimulation plus apyrase;  $0.67 \pm 0.06$ , N9 cells, static conditions;  $0.82 \pm 0.08$ , N9



**FIGURE 5.** Astrocyte-derived ATP induces vesicle shedding and IL-1 $\beta$  release from microglial cells. **A**, ELISA evaluation of IL-1 $\beta$  levels in the supernatants of N9-astrocyte cocultures and pure N9 cultures maintained in static condition or mechanically stimulated with or without apyrase (one-way ANOVA, post hoc Dunn's method,  $p = 0.003$ ,  $n = 3$ ). **B**, Merged differential interference contrast and fluorescence images from an astrocyte-microglia coculture incubated with Annexin V<sup>FITC</sup> following the mechanical stimulation of a single astrocyte present in the field by a patch pipette. Note the presence of annexin V-positive blebs on the microglial cell adjacent to the stimulated astrocyte. **C**, Quantitative analysis of annexin V-positive N9 cells present in N9-astrocyte cocultures maintained in static condition or mechanically stimulated by an orbital shaker in the presence or in the absence of the ATP degrading enzyme apyrase. The mechanical stimulus significantly increases the number of annexin V-positive N9 cells present in cocultures but not in pure N9 cultures.



cells, mechanical stimulation; one-way ANOVA, post hoc Dunn's method,  $p = 0.006$ ,  $n = 3$  Fig. 5C). Note that the presence of apyrase during mechanical stimulation almost completely inhibited bleb formation (Fig. 5C), further supporting a key role of astrocyte-derived ATP in the observed blebbing phenomenon.

## Discussion

It is currently assumed that, in the CNS, IL-1 $\beta$  is mainly expressed and released from microglia, although there is evidence that also astrocytes and neurons may contribute to IL-1 $\beta$  production, particularly in the late phase after excitotoxicity (18, 29).

In the present study we directly evaluated the specific contribution of astrocytes and microglia to the cytokine release. We demonstrate that 1) in hippocampal primary astrocyte-microglia cocultures, microglia is indeed the cell type which mainly releases IL-1 $\beta$ , 2) ATP released from astrocytes is the endogenous stimulus which leads to the cytokine secretion from microglial cells, and 3) astrocyte-derived ATP causes IL-1 $\beta$  secretion by inducing the shedding from microglial surface of membrane vesicles which contain all the machinery necessary for the cytokine processing, including P2X<sub>7</sub>Rs.

Pro-IL-1 $\beta$  is also expressed by primary hippocampal astrocytes, primed with LPS, although these cells fail to release the cytokine upon ATP stimulation. Because both astrocytes and microglia bear P2X<sub>7</sub>Rs (30, 31), which are essential for the cytokine release (2) further studies will be required to elucidate the molecular mechanisms underlying the different behavior of hippocampal astrocytes and microglia regarding IL-1 $\beta$  secretion. Lack of IL-1 $\beta$  release from astrocytes might result from differences in caspase-1 activation and/or cytokine processing, possibly due to insufficient intracellular Ca<sup>2+</sup> concentration increases and K<sup>+</sup> drop, following P2X<sub>7</sub> activation (F. Bianco and C. Verderio, unpublished observations).

The main finding of this study is that the release of at least a fraction of IL-1 $\beta$  from microglia takes place, upon ATP stimulation, through the shedding of vesicles from the plasma membrane. Shed vesicles appear to be the site of IL-1 $\beta$  processing.

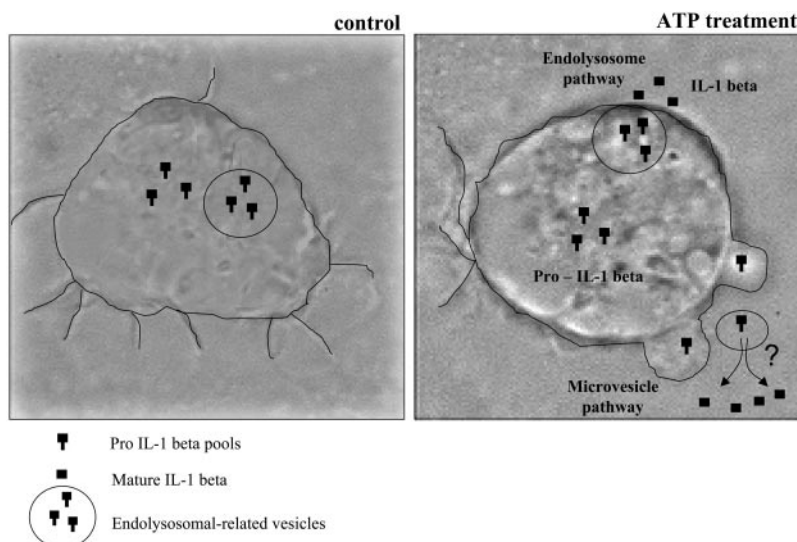
Different populations of membrane vesicles which can be released from cells in the extracellular environment have been described so far, including exosomes, microvesicles, and apoptotic vesicles. Exosomes originate from exocytosis of endolysosome-related multivesicular bodies and represent a population of vesicles homogenous in size and shape (40–80 nm) (32). Microvesicles and apoptotic vesicles represent instead a heterogeneous population of vesicles relatively larger than exosomes (100 nm to 1  $\mu$ m)

which bud directly from the plasma membrane of either viable cells or cells undergoing apoptosis (26). Exosomes and microvesicles/apoptotic vesicles are biochemically distinct; the latter population have PS exposed to the outer cell membrane (11, 26) carry some cytoskeletal elements (26) and can bear both nuclear and ER proteins (33, 34). Exosomes instead do not have PS exposed to the outer membrane leaflet and carry a selected subset of protein including rab-7 (26, 35).

Our video microscopy data indicate that ATP stimulation induces in microglia the shedding of membrane vesicles of heterogeneous diameter, which detach from the membrane in the presence of extracellular calcium. Because calcium chelation slightly delayed formation of ATP-induced membrane blebs, but markedly inhibited vesicle shedding, our results seem to dissociate the P2X<sub>7</sub>-dependent formation of membrane blebs from the release of vesicles. Bleb formation and vesicle shedding appear to be distinct parallel pathways both initiated by P2X<sub>7</sub> activation. Shed vesicles carry the plasma membrane soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) protein SNAP-23, have PS exposed to the outer cell membrane, do not express lysosomal markers, including rab-7, lamp-1, and cathepsin D, but bear the ER marker ribophorin and some actin. Based on biochemical and morphological analysis, vesicles isolated from the supernatant of ATP-stimulated microglia have all the features of microvesicles/apoptotic vesicles. Given that microglia are able to recover from ATP challenge and do not necessarily undergo apoptosis, and because no caspase-3 activity is detectable inside isolated vesicles (F. Bianco and C. Verderio, unpublished observations), we exclude these formations to be apoptotic vesicles. Accordingly, it has been reported that caspase-3 activation is not a necessary prerequisite for vesicle formation induced by ATP, although it plays a necessary role in blebbing induced by apoptosis (13).

The experiments described in this study indicate that shed vesicles contain pro-IL-1 $\beta$  which is cleaved in the active form and released into the extracellular environment in an ATP- and P2X<sub>7</sub>-dependent manner. These findings strongly suggest that IL-1 $\beta$  processing could proceed in shed vesicles, which indeed contain caspase-1 both as proenzyme and as active form. The presence of caspase-1 activity in shed vesicles is in agreement with previous reports showing that these vesicles act as carriers of proteases (36, 37), which can locally process their substrates (38). Our results point to a crucial involvement of the P2X<sub>7</sub>Rs in the mechanism mediating the cytokine processing and release from shed vesicles, although the

**FIGURE 6.** Model for IL-1 $\beta$  cleavage and release from shed vesicles and from cell surface. Two pathways of IL-1 $\beta$  processing and release may coexist in microglia: one pathway, mediated by the exocytosis of endolysosome-related vesicles, could take place at the cell surface (6); the other takes place at shed vesicles although the mechanism underlying the efflux of the cytokine from the vesicles remains to be defined (this study).



mechanism underlying the efflux of the cytokine from the vesicles remains to be defined. Vesicle disruption does not significantly contribute to the release of the cytokine, differently from what is described in fibroblasts where vesicle lysis mediates the release of fibroblast growth factor-2, another protein lacking a conventional secretory peptide which is released by vesicle shedding (32).

Globally, our data support and complement results obtained in monocytes by MacKenzie et al. (11), which indicated the existence of a vesicular pathway for IL-1 $\beta$  release by vesicle shedding. However, the observation that vesicle shedding can occur in resting microglia, which does not release IL-1 $\beta$ , supports the idea that vesicle shedding does not represent a secretory mechanism selective for the cytokine, but rather a secretory pathway possibly shared by other releasable factors, which is used by IL-1 $\beta$  when produced in activated microglia. Given that P2X<sub>7</sub>R activation in microglia triggers the synthesis and release of other cytokines like TNF (39) and proteases as well as their precursors, including plasminogen (40), it would be of interest to investigate whether ATP-induced shed vesicles can also mediate the release of such factors. Furthermore, vesicle shedding, through the release of cell surface components, including membrane receptors (41–43), can serve functions other than secretion of soluble factors. In this regard, we clearly demonstrate the presence of P2X<sub>7</sub>Rs on isolated vesicles, thus indicating that vesicles shedding might represent a mechanism to remove P2X<sub>7</sub>Rs from the cell surface in response to astrocyte-derived ATP signaling. From this point of view the shedding process could represent a defense strategy of microglia against apoptotic insults, produced by excessive or repetitive stimulation by astrocyte-derived ATP. Removal from the cell surface of functional P2X<sub>7</sub>Rs could facilitate microglial survival and avoid P2X<sub>7</sub>-mediated microglial apoptosis (3).

Based on the amount of IL-1 $\beta$  detected in Triton X-100-treated supernatant at 10-min stimulation, when negligible levels of the cytokine are present in vesicle-free supernatant, we estimated the fraction releasable by vesicle shedding to be ~27% of total cellular content. This estimation is in agreement with results from a previous study (2), which reported a releasable IL-1 $\beta$  fraction of ~25% in these cells. Based on these findings, one could speculate that vesicle shedding may be the predominant mechanism of IL-1 $\beta$  secretion in microglia. However, different pathways of IL-1 $\beta$  release might coexist in these cells (Fig. 6). This possibility has been already described for monocytes, where vesicle shedding (11) and exocytosis of endolysosome-related vesicles (6) have been shown to account for ~12 and 30% of total IL-1 $\beta$  cellular content, respectively. Further studies are needed to determine the possible contribution of the endolysosome pathway to IL-1 $\beta$  secretion in microglia, although the lack of inhibition of IL-1 $\beta$  release by AACOCF3 (F. Bianco and C. Verderio, unpublished observations), a PLA<sub>2</sub> blocker which strongly inhibits the exocytosis of secretory lysosomes (6), plays against a major contribution of the endolysosomal pathway in microglia.

Although vesicle shedding is most likely a nonspecific and non-unique pathway for IL-1 $\beta$  release, it is extremely important to understand the physiological relevance of IL-1 $\beta$  release from microglial cells by this mechanism. Shed vesicles, containing packages of IL-1 $\beta$ , can deliver the cytokine in possible proximity to IL-1 $\beta$ Rs present on target cells. Furthermore, given that IL-1 $\beta$  release is enhanced by ATP, the cytokine might be released from shed vesicles when they impact local high concentration of the purine, presumably in close proximity of releasing astrocytes, thus avoiding dilution of the cytokine in the extracellular environment. This might also turn out to be of crucial importance for the onset of a detrimental effect of IL-1 $\beta$  toward degenerating cells, which are known to release large amounts of ATP. Therefore, IL-1 $\beta$  re-

lease from microglia can represent a backward signal sent by microglial cells in response to ATP-mediated astrocyte-to-microglia signaling.

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## Disclosures

The authors have no financial conflict of interest.

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