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ATP Mediates Calcium Signaling Between Astrocytes and Microglial Cells: Modulation by IFN-γ

Claudia Verderio and Michela Matteoli

Calcium-mediated intercellular communication is a mechanism by which astrocytes communicate with each other and modulate the activity of adjacent cells, including neurons and oligodendrocytes. We have investigated whether microglia, the immune effector cells involved in several diseases of the CNS, are actively involved in this communication network. To address this issue, we analyzed calcium dynamics in fura-2-loaded cocultures of astrocytes and microglia under physiological conditions and in the presence of the inflammatory cytokine IFN-γ. The intracellular calcium increases in astrocytes, occurring spontaneously or as a result of mechanical or bradykinin stimulation, induced the release of ATP, which, in turn, was responsible for triggering a delayed calcium response in microglial cells. Repeated stimulations of microglial cells by astrocyte-released ATP activated P2X7 purinergic receptor on microglial cells and greatly increased membrane permeability, eventually leading to microglial apoptosis. IFN-γ increased ATP release and potentiated the P2X7-mediated cytolytic effect. This is the first study showing that ATP mediates a form of calcium signaling between astrocytes and microglia. This mechanism of intercellular communication may be involved in controlling the number and function of microglial cells under pathophysiologic CNS conditions.


Resident microglia are CNS immune effector cells that participate in many different diseases (1, 2). Under pathologic conditions they change their morphology, up-regulate a number of surface molecules, and acquire the features of cytotoxic, phagocytic cells. Although the phenomenology of their activation has been well documented, the signals by which they interact with their environment are still elusive (2). Microenvironment plays a key role in CNS immunopathogenetic events, and the interactions between microglia and lymphocytes or other immunocompetent cells probably play a pivotal role in the development of CNS inflammation (3). Identifying the key signals governing the interactions between microglial cells and their surroundings is of paramount importance for understanding both normal and pathologic functioning of the nervous system.

ATP is a major factor mediating intercellular communication in the immune and nervous systems and triggers a variety of strikingly different biological effects (4–7). In the brain it is considered to be the dominant extracellular messenger for astrocyte-to-astrocyte calcium-mediated communication. Astrocytes release ATP upon mechanical stimulation (8) or glutamatergic receptor activation (9) and respond to ATP with a propagating wave of intracellular calcium ([Ca2+]i) increases (8), a process that is thought to serve as a long-range signaling system in the CNS (10, 11).

Functionally active purinergic receptors have been detected in cultured and in situ microglial cells (12–17), thus suggesting the possibility that ATP may also act in astrocyte-to-microglia communication. In this study we demonstrate the existence of an ATP-mediated calcium signaling mechanism between astrocytes and microglial cells that is increased in the presence of the inflammatory cytokine IFN-γ and eventually leads to microglial apoptosis.

Materials and Methods

Cell cultures

Hippocampal mixed glia cultures from embryonic rat pups (embryonic day 18) were obtained using previously described methods (18). The cultures were grown in MEM supplemented with 20% FCS and 5.5 g/L glucose. The astrocytic and microglial components of the cultures were determined by means of immunostaining for glial fibrillar acidic protein (GFAP) and CSF-1R. The purified microglia were harvested by shaking 3-wk-old cultures, seeded on glass coverslips, and cultured in the same medium.

Fura-2 videomicroscopy

The cultures were loaded with 5 μM fura-2 pentacetoxy-methylester in Krebs-Ringer solution buffered with HEPES (KRH; 150 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 2 mM CaCl2, 10 mM glucose, and 10 mM HEPES/NaOH, pH 7.4) for 1 h at 37°C, washed in the same solution to allow de-esterification of the dye, and transferred to the recording chamber of an inverted microscope (Axiovert 100; Zeiss, New York, NY) equipped with a calcium-imaging unit. A modified CAM-230 dual wavelength microfluorometer (Jasco, Tokyo, Japan) was used as a light source for the assays. The experiments were performed at room temperature (24–25°C) using an Axon Imaging Workbench 2.2 equipped with a PCO Super VGA SensiCam (Axon Instruments, Foster City, CA). A single astrocyte in the field was gently mechanical stimulated with a glass microelectrode to evoke intercellular calcium waves.

ATP measurements

Bioluminescence assay. ATP levels in the extracellular saline incubated 30 min with dishes containing a pure hippocampal astrocytic monolayer were measured using a luciferin/luciferase assay (Molecular Probes, Leiden, The Netherlands) and a luminometer (Lumat LB9501; Berthold, Nadowa, NH). The experimental samples were compared with a standard ATP curve created on the basis of saline samples containing known concentrations of ATP. Each sample was run in duplicate. Most of the samples were assayed within 5–10 min of collection; the others were frozen for subsequent ATP determination.
**Extracellular ATP bioassay.** One milliliter of extracellular saline incubated for 30 min with dishes containing a pure hippocampal astrocytic monolayer in the presence or in the absence of IFN-γ was split into two aliquots before testing on fura-2-loaded astrocytes. One aliquot was pre-treated with apyrase (30 U/ml) for 15 min before testing. Five hundred microliters of test solution or saline containing known concentrations of ATP were applied to astrocytes as ATP sensor cells during image acquisition, and the amplitude of the [Ca\(^{2+}\)] response was measured.

**Immunochemistry**

At the end of the recording session, cultures were fixed at room temperature with 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.12 M sucrose for 25 min. Fixed cells were permeabilized with detergent and labeled with anti-GFP monoclonal Abs (Sigma, Milan, Italy), and anti-CSF-R1 polyclonal Abs (Santa Cruz Biotechnology, Santa Cruz, CA), followed by fluorescein-anti-mouse Abs (Jackson ImmunoResearch, West Grove, PA) and rhodamine-conjugated anti-rabbit Abs (Jackson ImmunoResearch). The coverslips were mounted in 70% glycerol in phosphate buffer containing 1 mg/ml phenylendiamine. The images were acquired using a Bio-Rad MRC-1024 confocal microscope (Bio-Rad, Hercules, CA) equipped with LaserSharp 3.2 software. The morphological features of apoptosis were monitored by means of phase contrast microscopy and labeling with propidium iodide in PBS on cultures fixed in methanol precooled at −20°C.

**Chemicals**

Most of the chemicals (pyridoxalphosphate-6-azophenyl-2,4-disulfonic acid, tetrasodium (PPADS), ATP, bradykinin, apyrase, and 18 α-glycyrrhetinic acid) were obtained from Sigma. Fura-2-acetoxyethyl ester was purchased from Calbiochem (La Jolla, CA), and periodate-oxidized ATP was a gift from Prof. F. Di Virgilio (University of Ferrara, Ferrara, Italy).

**Results**

[Ca\(^{2+}\)] increases in astrocytes induce delayed [Ca\(^{2+}\)], responses in microglial cells

Primary cultures of embryonic hippocampal glial cells predominantly contain astrocytes, with a variable percentage of microglial cells. Microglial cells are characterized by their amoeboid shape and can be unambiguously distinguished from astrocytes because they are stained by Abs against the CSF receptor (CSF-1R; Fig. 1, A and B, red), but not by Abs against the GFAP cytoskeletal glial protein (green). The mixed cultures were loaded with the cell-permeant fura-2-acetoxyethyl ester fluorescent calcium dye and imaged using digital imaging techniques in a static bath. Gentle mechanical contact between a patch pipette and the surface of a single astrocyte (pseudocolor images in Fig. 1C, arrow) induced a propagating calcium wave in neighboring cells (Fig. 1C) that were identified as astrocytes or microglia on the basis of morphological criteria or CSF-1R and GFAP immunostaining at the end of the recording session (Fig. 1C, inset). Analysis of the kinetics of [Ca\(^{2+}\)] changes in the stimulated astrocytes and in two adjacent microglia cells revealed a transient, delayed [Ca\(^{2+}\)], response in the microglial cells (Fig. 1D).

To confirm that the delayed [Ca\(^{2+}\)], response in microglia was a consequence of increased [Ca\(^{2+}\)], in astrocytes, cocultures were stimulated with 1 μM bradykinin, a widely used stimulus for selectively evoking increased [Ca\(^{2+}\)], in astrocytes (19). Bradykinin did not cause any [Ca\(^{2+}\)], changes in the purified microglial cells shaken from mixed cultures and plated onto glass coverslips (Fig. 1E, bottom panel), but a large increase in [Ca\(^{2+}\)], was induced in the majority of cocultured astrocytes and microglial cells (Fig. 1E, top panel). Whereas bradykinin application induced a prompt [Ca\(^{2+}\)], response in astrocytes, [Ca\(^{2+}\)], increases occurred several seconds later in microglia (50 ± 20 s; mean ± SE; n = 12). Efficient propagation of calcium signaling between astrocytes and microglia was also observed in cultures characterized by spontaneous [Ca\(^{2+}\)], oscillations (Fig. 1F), a phenomenon that may depend on cycles of calcium release and uptake from intracellular stores (10, 11, 20, 21). Analysis of several fields containing both astrocytes and microglial cells (n = 31) revealed that spontaneous astrocytic [Ca\(^{2+}\)], oscillations were coupled to a delayed response in adjacent microglial cells (Fig. 1F).

**Delayed [Ca\(^{2+}\)], responses in microglial cells are mediated by ATP**

The delayed response of microglia triggered by spontaneous or evoked [Ca\(^{2+}\)], increases in astrocytes is consistent with a secondary response to a bioactive compound released by astrocytes upon [Ca\(^{2+}\)], elevations. Glutamate and ATP have recently been identified as diffusible factors, capable of inducing calcium waves among astrocytes (8, 22). As microglial cells from hippocampus lack calcium-permeable glutamate receptors (our unpublished observation) and express purinergic receptors permeable to calcium ions (15), we investigated whether ATP might be involved in intercellular calcium signaling between astrocytes and microglial cells. We found different lines of evidence indicating that this was the case. First, ATP was detected in the extracellular medium of bradykinin-stimulated and spontaneously oscillating astrocytes by means of the sensitive luciferase bioluminescence assay (not shown) and was also detected in the medium collected from mechanically stimulated astrocytes (8). Second, samples of extracellular medium taken from spontaneously oscillating astrocytes and gently added to fura-2-loaded astrocytes as ATP-sensor cells induced a [Ca\(^{2+}\)], response that was prevented by pretreatment of the conditioned medium with the ATP-degrading enzyme apyrase (Fig. 5C, right panel). Third, when the astrocytes in astrocyte-microglia cocultures were stimulated mechanically or with bradykinin, a potent inhibition of the propagation of the [Ca\(^{2+}\)], signal to microglial cells was recorded in the presence of either the P2X/P2Y purinergic receptor antagonist PPADS (50 μM) or apyrase (Fig. 2). The series of pseudocolor images in Fig. 2A shows an example of a mechanically induced calcium wave (Fig. 2A, patch pipette in position 1) that did not propagate to microglia in the presence of PPADS (Fig. 2A, arrows, see inset in image 64 for immunocytochemical identification) despite the efficient transmission of the calcium signal to adjacent astrocytes (Fig. 2A, images from 0–64 s). After extensive washing of PPADS, the calcium wave induced by mechanical stimulation of another astrocyte in the field (Fig. 2A, patch pipette shifted to position 2) did propagate to microglia according to the diffusion of the wave front (Fig. 2B, images from 0 to 31 s). The kinetics of [Ca\(^{2+}\)], changes in the two stimulated astrocytes (green traces) and in one microglial cell (red trace, positioned between 1 and 2) are shown in Fig. 2C. The calcium signal propagation from astrocytes to microglial cells was also potently inhibited in the presence of the ATP-degrading enzyme apyrase (Fig. 2D). Fig. 2E shows the quantitative analysis of PPADS and apyrase inhibition of the delayed response of microglia (81% inhibition in the presence of PPADS, n = 8; 66.5% inhibition in the presence of apyrase, with a 420% increase in the time to peak response, n = 13; controls, n = 14); a similar reduction in calcium signal propagation was produced by PPADS when increased astrocyte [Ca\(^{2+}\)], levels were evoked by bradykinin (Fig. 2E; 83.9% inhibition in the presence of PPADS, n = 9; controls, n = 12). Efficient propagation of the calcium signal between astrocytes and microglia was detected in the presence of a cocktail of glutamate receptor antagonists (100 μM 2-amino-5-phosphonovaleric acid, 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione, and 1 mM (S)-α-methyl-carboxyphenylglycine) or after 5- to 10-min incubation in the presence of the gap junction blocker 18α-glycyrrhetinic acid (10 μM; data not shown). These results indicate that [Ca\(^{2+}\)], increases in astrocytes induce the release of ATP, which, in turn, activates purinergic receptors in microglial cells.
FIGURE 1. Increased \([\text{Ca}^{2+}]_i\) in astrocytes induces delayed calcium responses in microglial cells. A and B, Double labeling of purified microglia (A) and mixed glial cultures (B) with Abs against CSF-R (red) and against GFAP (green). Calibration bars, 10 \(\mu\text{m}\) (A) and 22 \(\mu\text{m}\) (B). C, Series of pseudocolor images of fura-2-loaded mixed glial cultures taken 3, 5, 13, and 23 s after the mechanical stimulus (arrow). Inset, Double labeling of the same field with Abs against CSF-R (red) and against GFAP (green). Calibration bars, 20 \(\mu\text{m}\) (C) and 41 \(\mu\text{m}\) (inset). D, Temporal analysis of \([\text{Ca}^{2+}]_i\), changes in the stimulated astrocyte (green) and in the two adjacent microglial cells (red) shown in C. E, top panel, Temporal plot of \([\text{Ca}^{2+}]_i\), changes induced by 1 \(\mu\text{M}\) bradykinin in an astrocyte (green) and in an adjacent microglia (red). Note the delay in the increase in \([\text{Ca}^{2+}]_i\), in the microglial cell. E, bottom panel, No \([\text{Ca}^{2+}]_i\) changes are detectable in purified microglial cells upon bradykinin stimulation. F, Kinetics of spontaneous \([\text{Ca}^{2+}]_i\) oscillations in astrocytes (green) cocultured with microglia (red).
Activation of P2X7 receptors in purified microglial cells

Different concentrations of exogenous ATP were applied to purified microglia shaken from mixed glial cultures and plated onto glass coverslips. As shown in Fig. 3A (left panel), ATP (10 μM) triggered a [Ca^{2+} ] spike, followed, or not, by a [Ca^{2+} ] plateau, and returned to basal levels upon ATP removal. As previously described (15, 17), the increase in [Ca^{2+} ] presumably reflects two different mechanisms: the release of stored calcium (first spike) and calcium influx from the extracellular medium (second shoulder). The biphasic [Ca^{2+} ] response was completely prevented by 50 μM PPADS (data not shown), thus suggesting that the [Ca^{2+} ] response, triggered by micromolar amounts of ATP, is linked to the activation of PPADS-sensitive P2X/Y receptors. Addition of 1 mM ATP (Fig. 3A, left panel) greatly enhanced the duration of the spike and the plateau, with the latter lasting several minutes after ATP removal. The persistent increase in [Ca^{2+} ] was followed by a rapid decrease in the 340:380 fluorescence ratio, thus indicating a progressive increase in membrane permeability that led to the complete efflux of FURA2 from the cell (Fig. 3A, left panel, light blue trace). This decrease in the fluorescence signal was paralleled by the disappearance of the microglial cell profiles in the pseudocolour images, although the cells were still attached to the coverslips (data not shown).

Purinergic P2X7 receptors (23) are expressed in microglia (15, 24). When stimulated by prolonged or frequent agonist application, these ATP-gated ion channels form large transmembrane pores that are permeable to molecules of up to ~1000 Da in size (23), and, upon activation, allow the efflux of cytoplasmic water-soluble molecules such as fura-2-free acid into the extracellular medium (25). Oxidized ATP (oATP) is a rather selective blocker of P2X7 receptors. Preincubation of purified microglial cells for 1–2 h with 300 μM oATP did not significantly affect the [Ca^{2+} ] response induced by 10–20 μM ATP (Fig. 3A, right panel), but abolished the long-lasting increase in calcium and membrane permeability in 66.6% of the cells exposed to 1 mM ATP (Fig. 3A, right panel; n = 15). This indicates that microglia express P2X7 receptors that mediate fura-2 efflux once activated by millimolar amounts or repeated applications of micromolar amounts of ATP.
Interestingly, repetitive challenges with 10–20 μM ATP (two or three pulses of 1–3 min) resulted, after a several minute delay, in a sustained calcium plateau followed by an increase in membrane permeability that led to the complete efflux of fura-2 from the cell (Fig. 3B).

**FIGURE 3.** Activation of microglial P2X7 receptors in purified microglia and astrocyte-microglia cocultures. A. Kinetics of [Ca^{2+}]i changes in purified microglia upon the addition of 10 μM and 1 mM ATP in control cultures (A, left panel) and after 2-h treatment with oATP (300 μM; A, right panel). oATP significantly reduces the long-lasting increase in [Ca^{2+}]i, following 1 mM ATP addition. B, Kinetics of [Ca^{2+}]i changes in purified microglia upon repeated stimulation with 10 μM ATP. The two traces in A and B represent recordings from different microglial cells. C and E, Pseudocolor images of a fura-2-loaded mixed culture showing the efflux of the calcium dye from two distinct microglial cells (*). The brightfields in C and E were acquired at the end of the recording session. Calibration bar, 8.5 μm. D and F, Corresponding temporal plots of [Ca^{2+}]i changes in the marked microglia. G, Temporal analysis of [Ca^{2+}]i changes in a mixed culture, showing that a long-lasting [Ca^{2+}]i increase in microglia (blue trace) is triggered by spontaneous calcium elevations in the astrocyte (green trace). H, Plots of the cumulative percentages of permeabilized microglial cells vs time (minutes) in control conditions (green traces) and in the presence of PPADS (black trace) or oATP (light blue trace). The complete fura-2 efflux revealed by the rapid decrease in the 340:380 fluorescence signal was considered a parameter of cell lysis. Note that the P2X7 antagonist significantly increases the time required for microglial permeabilization.

**Activation of P2X7 receptors in microglial cells cocultured with astrocytes**

In microglial cells cocultured with astrocytes, long-lasting P2X7-mediated [Ca^{2+}]i increases occurred in the absence of exogenously applied ATP, but were triggered by the [Ca^{2+}]i oscillations that...
Astrocytes-released ATP mediates cytotoxicity in microglial cells

It has been reported that activation of P2X<sub>7</sub> receptors by exogenous ATP induces microglial cell death (24), because pore opening radically disrupts ionic homeostasis and causes the depletion of intracellular low weight metabolites. Fig. 4 shows that microglial cell death is produced by pharmacological treatments that increase astrocyte [Ca<sup>2+</sup>]<sub>i</sub> and enhance ATP release; 24-h treatment of astrocyte-microglia cocultures with repeated challenges of 1 μM bradykinin in KRH led to massive microglial death accompanied by chromatin condensation as revealed by the intercalation of propidium iodide in the DNA (Fig. 4B). The astrocytes present in the mixed glial cultures did not show any morphological changes typical of apoptosis (Fig. 4B) and had intact nuclei. The percentage of condensed nuclei in microglia associated with astrocytes was 73.5% in bradykinin-treated cultures; however, such degenerative changes occurred to a much lesser extent (18.4%) in the microglia present in the same coverslip but not directly in contact with astrocytes (Fig. 4A). The percentage of condensed nuclei in microglia associated with astrocytes in the absence of pharmacological treatments was 51.5%. Thus, ATP locally released by astrocytes, either spontaneously or upon stimulation, activates P2X<sub>7</sub> receptors, leading to the apoptotic death of microglial cells.

IFN-γ potentiates ATP-mediated calcium signaling between astrocytes and microglia

Application of IFN-γ to pure astrocyte cultures caused an average increase in the frequency of spontaneously occurring [Ca<sup>2+</sup>]<sub>i</sub> oscillations of 520% (n = 37 control; n = 42 IFN-γ-treated cells), with a preonset latency of 5–10 min (Fig. 5, A and B). The mean frequency of [Ca<sup>2+</sup>]<sub>i</sub> oscillations was derived from 30-min recordings made before and after application of IFN-γ. No significant changes in the frequency of [Ca<sup>2+</sup>]<sub>i</sub> oscillations were observed between the two 30-min recordings made in the absence of IFN-γ (data not shown).

The increased frequency of astrocytic [Ca<sup>2+</sup>]<sub>i</sub>, oscillations was associated with a 4- to 5-fold increase in the released ATP (Fig. 5C), which was detected in samples of extracellular medium incubated with astrocytes for 30 min before and after the addition of IFN-γ using the sensitive luciferase bioluminescence assay (Fig. 5C, left panel). The amount of ATP released by astrocytes was also determined using a biological assay (8) in which samples of extracellular medium taken before and after treatment with IFN-γ were gently added to fura-2-loaded astrocytes as ATP sensor cells. Although both samples of extracellular medium evoked a measurable [Ca<sup>2+</sup>]<sub>i</sub> response, the [Ca<sup>2+</sup>]<sub>i</sub> transients induced by the extracellular saline incubated with IFN-γ-treated astrocytes were significantly higher (see quantitation in Fig. 5C, right panel). Based on a standard dose-response curve of the calcium response amplitude vs different ATP concentrations, the actual ATP level in the extracellular medium was estimated to be 140 ± 16 nM in basal conditions and 650 ± 45 nM after IFN-γ treatment. The local concentration of ATP at the releasing astrocyte was probably higher, as microglial cells do not respond to exogenous application of ATP <10 μM. The [Ca<sup>2+</sup>]<sub>i</sub> responses were prevented when the conditioned medium was treated with apyrase (30 U/ml for 10–15 min), thus indicating that ATP is the bioactive compound released in the medium and is responsible for the observed effects.

IFN-γ potentiates P2X<sub>7</sub> receptor activation and microglial apoptosis

In line with the enhanced stimulation of ATP release from astrocytes, IFN-γ considerably shortened the time of activation of P2X<sub>7</sub> receptors in microglial cells cocultured with astrocytes (Fig. 6A). The treatment of mixed glial cells cultures with IFN-γ for 24 h exacerbated the apoptotic death of microglial cells, as revealed by
chromatin condensation of the nuclei stained with propidium iodide. The quantitative results are shown in Fig. 6B; 51.5% of the microglial cells lying in strict contact with astrocytes underwent apoptosis under control conditions, but a significantly higher proportion of microglial cells (88.8%) showed typically apoptotic morphological changes after IFN-γ treatment. On the contrary, no significant apoptotic changes were detected in the microglial cells present in the same coverslip but not directly in contact with astrocytes, indicating a local cytolytic effect of astrocyte-released ATP in the cultures.

Discussion

Calcium-mediated signaling is one of the mechanism by which CNS cells communicate with and modulate the activity of adjacent cells (11, 26). Astrocytes represent the central cellular element in this network insofar as their large diversity of membrane receptors allows them to sense external stimuli mainly deriving from neurons (21). In response to environmental inputs, astrocytes show oscillations in \([Ca^{2+}]_i\) oscillations in astrocytes recorded 30 min before and after the addition of IFN-γ. The traces were recorded from distinct astrocytes in the field. B, Quantitative analysis of the frequency of \([Ca^{2+}]_i\) oscillations in astrocytes recorded 30 min before and after the addition of IFN-γ. Values are the mean ± SE derived from five different fields (seven or eight cells per field) normalized in relation to controls. C, Quantitative analysis of ATP levels in KRH conditioned by pure astrocyte cultures for 30 min with or without IFN-γ. ATP was detected using the luciferin/luciferase bioluminescence assay (left) or a bioassay (right; see text for details). Values represent percent changes normalized to controls (±SE) and are derived from three independent experiments.

FIGURE 5. IFN-γ increases the frequency of spontaneous \([Ca^{2+}]_i\) oscillations and enhances ATP release from pure astrocytic cultures. A, Representative traces of \([Ca^{2+}]_i\) kinetics in pure astrocyte cultures exposed to 200 U/ml IFN-γ. Note that IFN-γ induces or potentiates the frequency of \([Ca^{2+}]_i\) oscillations in astrocytes. The traces were recorded from distinct astrocytes in the field. B, Quantitative analysis of the frequency of \([Ca^{2+}]_i\) oscillations in astrocytes recorded 30 min before and after the addition of IFN-γ. Values are the mean ± SE derived from five different fields (seven or eight cells per field) normalized in relation to controls. C, Quantitative analysis of ATP levels in KRH conditioned by pure astrocyte cultures for 30 min with or without IFN-γ. ATP was detected using the luciferin/luciferase bioluminescence assay (left) or a bioassay (right; see text for details). Values represent percent changes normalized to controls (±SE) and are derived from three independent experiments.

FIGURE 6. IFN-γ effects on astrocyte-to-microglial cell signaling. A, Plot of the cumulative percentages of permeabilized microglial cells cocultured with astrocytes vs time (minutes) in control conditions (○, △, □) and in the presence of IFN-γ (●, ▲, ★). Note that the cytokine significantly shortens the time required to induce microglial cell lysis. B, Quantitative analysis of the percentages of apoptotic microglial cells in mixed cultures after 15-h incubation in control KRH or in the presence of IFN-γ. Values are the mean ± SE derived from two independent experiments. Note that no significant degenerative apoptotic changes were observed in the majority of microglial cells present in the same coverslip and growing in the absence of adjacent astrocytes.

Discussion

Calcium-mediated signaling is one of the mechanism by which CNS cells communicate with and modulate the activity of adjacent cells (11, 26). Astrocytes represent the central cellular element in this network insofar as their large diversity of membrane receptors allows them to sense external stimuli mainly deriving from neurons (21). In response to environmental inputs, astrocytes show oscillations in \([Ca^{2+}]_i\) levels and propagate calcium signals to each other over long distances by means of calcium waves (10). Astrocytic calcium signals also propagate to other neighboring cell types, including neurons (19, 27), oligodendrocytes (28), and other neuroglial element (29), thereby generating an interconnected communication network that may modulate different CNS functions. Homo- and heterotypical cell-to-cell calcium signaling involves the diffusion of intracellular messengers (calcium and inositol trisphosphate) via gap junctions (27, 30, 31), and the release of extracellular messengers (ATP and glutamate) (8, 19, 22). Despite the increasing number of reports concerning the mechanisms of calcium-mediated intercellular signaling in the CNS, no evidence has previously been provided concerning the possible involvement of microglia, the CNS immune effector cells, in this heterocellular network. The results of our study demonstrate that calcium signaling events can be readily transmitted from astrocytes to microglial cells and provide evidence that astrocyte-to-microglia communication may have important implications for the pathophysiological functioning of the CNS.

Our results indicate that ATP is involved in the communication between astrocytes and microglial cells. As revealed by a biological assay and the luciferase bioluminescence assay, spontaneously oscillating and stimulated astrocytes release ATP in the extracellular medium. Furthermore, the purinergic antagonist PPADS (but not glutamate antagonists) potently inhibits the \([Ca^{2+}]_i\) transient induced in microglial cells as a result of mechanical stimulation of single astrocytes or bradykinin application. Finally, the ATP-degrading enzyme apyrase blocks the biological activity of the released mediator in the collected medium and inhibits propagation of the calcium wave to microglial cells.

This blocking action of purinergic antagonists also suggests that the transfer of calcium signaling between astrocytes and microglial
cells is entirely due to an extracellular messenger. Astrocyte-microglia intercellular communication mediated by gap junctions is unlikely because microglial cells are not dye-coupled with astrocytes (28). Furthermore, we observed that astrocytic calcium signaling efficiently propagates to microglial cells in the presence of the gap junction blocker 18α-glycerophosphoric acid. Given that 18α-glycerophosphoric acid as well as other gap junction blockers may also block the release of ATP controlled by connexins (32), our data support the existence of a calcium-dependent, gap junction blocker-insensitive release of ATP from astrocytes. How ATP crosses the membrane of astrocytes is still unknown. The existence in astrocytes of typical secretory granules undergoing regulated secretion has been recently reported (33), and a number of synaptic proteins for regulated secretion in neuronal cells have been identified in glial cells (34–36). The association of these proteins with ATP-storing organelles in astrocytes (36), along with the partial sensitivity of ATP release to clostridial toxins (S. Coco, M. Matteoli, and C. Verderio, unpublished observations) suggest that ATP in astrocytes is stored in intracellular vesicles that undergo regulated secretion.

The main finding of this study is that astrocyte-released ATP mediates a paracrine activation of microglial P2X receptors that triggers a perturbation of calcium homeostasis and finally leads to microglial cell death. This is consistent with previously reported data showing that P2X receptor activation by exogenously administered ATP mediates cytolysis in mouse as well as in human macrophages and microglial cells (27, 37–40). Although other P2X receptor subtypes can form a pore permeable to ethidium dyes (41), the pore is entirely due to an extracellular messenger. Astrocyte-microglia contact is maintained by gap junctions (42), and the plasma membrane or induction of an autocrine/paracrine loop resulting in an even higher extracellular ATP concentration, able to activate P2X receptors.

\[ \text{[Ca}^{2+}]_{i} \text{, changes, which occur spontaneously (20) or in response to different kinds of stimuli (8, 9) in cultured astrocytes, have been shown to occur in brain as a consequence of neuronal activation and glutamate release (26). Alterations of calcium homeostasis in astrocytes have been suggested to occur in specific pathophysiological conditions of the CNS (43). Interestingly, we have found that the inflammatory cytokine IFN-γ increases the frequency of spontaneous calcium oscillations in glial cells. IFN-γ receptor stimulation has been previously associated with elevation of cAMP, elevations of inositol trisphosphohate that cause release of calcium from stores, and protein kinase C activation in cells of the immune system and astrocytes (44). The effect of IFN-γ on astrocyte calcium homeostasis potentiates astrocyte-microglia communication, with the enhanced ATP release from astrocytes presumably accounting for the increased apoptosis of microglial cells. This finding adds to the reported up-regulation of P2X receptors caused by prolonged IFN-γ treatment in human macrophages and mouse microglial cells (38, 25).

The activation of P2X<sub>1</sub> receptors induces IL-1β release from microglia (15, 45). It has recently been reported that IL-1β significantly down-regulates gap junction connectivity among astrocytes and also potentiates interastrocyte calcium signaling mediated by the extracellular messenger ATP (46). Microglial cells activated by ATP released from astrocytes upon IFN-γ treatment can therefore feed back to glial cells via IL-1β and thus activate a paracrine loop, sustaining further ATP release.

The repeated secretion of ATP from astrocytes and the apoptotic response in microglial cells may represent a homeostatic mechanism for controlling the number of microglial cells in pathophysiological conditions of the CNS. It is interesting to note that an increased number of microglial cells undergoing apoptosis has been reported in inflammatory demyelinating diseases, including experimental autoimmune encephalomyelitis in rats (47, 48). In acute multiple sclerosis (MS) plaques, in particular, microglia and macrophages represent a relevant percentage (~60%) of the TUNEL-positive cells (49, 50). It is noteworthy that IFN-γ has been selectively detected in active plaques, where it is predominately found on astrocytes (51). As the interaction between microglia and T cells is important in the development of CNS inflammation, the apoptosis of microglia could be related to the development and progression of MS, as it has been shown that Ag-stimulated T cells undergo apoptosis in the presence of microglial cells (52–54). By acting locally to kill activated T cells, microglia seem to be a component of the so-called immunological brain barrier (3) and may contribute to the down-regulation of immunopathologic processes in the brain. Our data showing that in the presence of the inflammatory cytokine IFN-γ, astrocytes induce extensive microglial apoptosis due to the release of ATP suggest that this mechanism may operate during MS inflammation and may thus represent a potential target for therapeutic strategies.

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