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ATP Released from Astrocytes Mediates Glial Calcium Waves

Peter B. Guthrie,[1] Joshua Knappenberger,[1] Menahem Segal,[2] Michael V. L. Bennett,[3] Andrew C. Charles,[4] and S. B. Kater[1]

[1]Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, Utah 84132, [2]Department of Neurobiology, Weizmann Institute, Rehovot 76100, Israel, [3]Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461, and [4]Department of Neurology, University of California, Los Angeles, School of Medicine, Los Angeles, California 90024

Calcium waves represent a widespread form of intercellular communication. Although they have been thought for a long time to require gap junctions, we recently demonstrated that mouse cortical astrocytes use an extracellular messenger for calcium wave propagation. The present experiments identify ATP as a major extracellular messenger in this system. Medium collected from astrocyte cultures during (but not before) calcium wave stimulation contains ATP. The excitatory effects of medium samples and of ATP are blocked by purinergic receptor antagonists and by pretreatment with apyrase; these same purinergic receptor antagonists block propagation of electrically evoked calcium waves. ATP, applied at the concentration measured in medium samples, evokes responses that are qualitatively and quantitatively similar to those evoked by those medium samples. These data implicate ATP as an important transmitter between CNS astrocytes.

Key words: glia; astrocytes; calcium waves; ATP; extracellular signal; suramin; apyrase; purinergic; electrical stimulation

Intercellular calcium waves, i.e., rises in intracellular free calcium that propagate between neighboring cells, occur widely among different cell types throughout the animal kingdom (Cornell-Bell et al., 1990; Boitano et al., 1992; Enomoto et al., 1992; Demer et al., 1993; Nathanson et al., 1995; Cao et al., 1997; Young et al., 1996; Jørgensen et al., 1997; Newman and Zahs, 1997). Although we are at an early stage of understanding the function of such waves, one must consider that glial calcium waves may provide an information-processing system operating in parallel with neuronal circuits within the nervous system. There is clear evidence of interaction between glial calcium waves and neurons; neuronal activity can directly evoke glial calcium waves (Dani et al., 1992), and glial calcium waves can directly evoke calcium transients and electrical activity in neurons (Nedergaard, 1994; Parpura et al., 1994; Hassinger et al., 1995; Newman and Zahs, 1998). Understanding how glial calcium waves could contribute to information processing requires an understanding of the mechanisms underlying calcium wave propagation.

For some years, glial calcium waves have been thought to propagate through gap junctions (Boitano et al., 1992; Charles et al., 1993; Sanderson et al., 1994; Sneyd et al., 1994, 1995). We demonstrated recently that an extracellular communication system can provide a dominant path for glial calcium wave propagation (Hassinger et al., 1996), because calcium waves can propagate between physically separated astrocytes, and the extent and direction of calcium wave propagation are significantly influenced by movement of the extracellular medium. Two subsequent publications now confirm that astrocytes do not absolutely require functional gap junction coupling for calcium wave propagation (Guan et al., 1997; Naus et al., 1997). Taken together, the newer literature supports the idea that substance(s) released from astrocytes can activate receptor systems on adjacent astrocytes, evoking release of additional excitatory substance(s) (either the same or different compounds) and thus producing a propagating wave of activity. Although these studies have demonstrated the existence of an extracellular communication pathway, the messenger (or messengers) underlying this extracellular communication have not been identified.

The experiments reported here show that ATP is the primary active messenger in this extracellular communication system. The first experiments confirmed that calcium waves will cross a cell-free area. Neutrophils were used to demonstrate that release of an extracellular messenger is associated with the calcium wave. Next, we determined that medium collected during calcium wave stimulation contained an excitatory substance. The excitatory material was ATP. (1) Purinergic antagonists blocked the excitatory effect of the collected medium, (2) treatment of samples with the degradative enzyme apyrase eliminated biological activity, and (3) the sensitive luciferase bioluminescence assay demonstrated the presence of ATP in collected samples. In addition, local application of ATP evoked propagating calcium waves. Finally, experiments demonstrated that purinergic antagonists blocked propagation of electrically evoked calcium waves, indicating that extracellular ATP is required for normal calcium wave propagation in this system.

MATERIALS AND METHODS

Cell culture. Glia from postnatal day 1–5 mouse pups were obtained using the methods described previously for rat pups (Hassinger et al., 1996). Briefly, the cortical hemispheres were removed, cleaned, and dissociated by trypsinization (0.2%; Life Technologies, Gaithersburg, MD) and mechanical trituration. Cells were plated on poly-L-lysine–coated glass coverslips, in culture flasks, or in 24-well plates (2 cm^2/well) at 50,000 cells/cm^2 and were maintained in growth medium [MEM (Life Technologies), 10% fetal calf serum (HyClone, Logan, UT), penicillin and streptomycin (Life Technologies), essential amino acids (Life Technolo-

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Correspondence should be addressed to Dr. Stanley B. Kater, Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT 84132.

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gies), and nonessential amino acids (Life Technologies)] with one to two media changes per week. Cultures were used at 5–15 d *in vitro*. All experiments were performed in observation saline (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 3 mM $CaCl_2$, 10 mM HEPES, and 25 mM glucose, pH 7.25) at room temperature.

Astrocytes passaged from confluent monolayers were used in several experiments. After enyzmatic treatment [0.05% trypsin + 0.53 mM EDTA (Life Technologies)] at 37°C for 10 min, astrocytes were washed off the culture surface, plated onto glass coverslips as described above, and used after 5–15 d. No differences were observed between passaged and primary cultured astrocytes.

Mobile reporter astrocytes were prepared from confluent cultures. Astrocytes were preloaded with fluo-3 and detached from the substrate using divalent cation-free medium + EGTA (2 mM) for 30 min. These astrocytes were then added en masse to another astrocyte culture by Pasteur pipet.

Imaging and data analysis. Intracellular calcium was monitored using the fluorescent indicator fluo-3 (Molecular Probes, Eugene, OR). Cultures were loaded with 5 μM fluo-3 AM for 50–60 min at 37°C, rinsed three times in observation saline solution, and allowed to de-esterify for an additional 30 min before use (Hassinger et al., 1996). Fluorescent images (100 msec exposures) were acquired using a 10×, 0.5 numerical aperture (NA) or a 20×, 0.75 NA objective and a Photometrics-cooled CCD camera. A mercury light source with a computer-controlled shutter was used for illumination. Fluorescence intensity was analyzed with IPLab software (Signal Analytics) on a Macintosh Power personal computer.

Sequences of images were acquired at 1–3 sec intervals. A control image (F_0) was based on 5–20 images collected before electrical stimulation. Changes in calcium concentrations within the astrocytes were monitored by changes in the fluorescence intensity (F) relative to the control image. A positive astrocytic response was recorded if relative fluorescence intensity (F/F_0) in at least one-half of the pixels for that cell exceeded 1.25. Astrocytes participating in the calcium wave were identified on the basis of both temporal and spatial correlation with respect to both the stimulation site and other astrocytes participating in the calcium wave. The calcium response evoked by extracellular message samples was measured as the number of astrocytes exceeding criterion. All experiments used yoked control cultures from the same plating, containing astrocytes at a similar density (the SD was ~5% of the mean number of cells in a field).

Human neutrophils were isolated from venous blood using dextran sedimentation and centrifugation through Ficoll-Hypaque, followed by removal of red blood cells by hypotonic lysis. Neutrophils were loaded with fura-2 AM (1 μM for 30 min). Image pairs were acquired with 350 and 380 nm excitation filters and were expressed as a ratio after background subtraction. Calcium concentrations were determined from the ratio images as described previously (Guthrie et al., 1991).

Astrocyte calcium wave stimulation. Propagating waves of elevated glial calcium were evoked electrically by extracellular stimulation using a saline-filled glass pipet with a tip diameter of 5–10 μm. The electrode was placed 10 μm above a target cell. Stimulation consisted of bipolar voltage pulses (40–80 V; 2 msec in total duration; 17 Hz; total stimulus duration, 3–8 sec) generated by a Grass SD9 stimulator. The stimulus voltage for a given stimulation pipet that reproducibly evoked astrocytic calcium waves was determined at a remote test site, many millimeters from the experimental sites in the same culture dish. Because electrically evoked calcium waves propagated for an average of 360 μm, these evoked calcium waves did not spread to the regions subsequently used for experiments. This voltage was used for stimulation of all fields in that culture dish. With this method, we obtained up to 10 successive responses from the same initiation site. If a calcium wave occurred, it began during the stimulation period; a fixed stimulation period was used for each data set. Electrical stimulation seldom resulted in any observable damage to the stimulated cell. Only fields in which the stimulated cell returned to near baseline fluorescence values were included in this report.

Microcollection of extracellular message samples. We have developed a system for collecting small volumes (<1 μl) of medium during an evoked calcium wave. A collection pipet (a patch pipet with a tip 3–5 μm in diameter) was produced using a standard patch pipet-pulling program on a Sutter Instruments P-2000 Laser Based Micropipet Puller (Novato, CA). The collection volume was determined with a 2.5 μl Hamilton syringe mounted in a screw-driven syringe holder; small rotations of the screw advanced or retracted the syringe piston. The entire system was oil-filled to provide reliable pressure transmission for precise volume control. For the experiments described in this report, the collection pipet tip was placed 10 μm above the surface of the astrocyte monolayer. An astrocyte two to three cells away from the collection pipet was stimulated electrically. As the calcium wave passed under the collection pipet (as monitored by fluo-3 fluorescence intensity), a predetermined volume was sucked into the tip of the collection pipet. The microscope stage was then moved to a distant field where astrocytes had not been stimulated either directly or indirectly by calcium wave propagation. The collected material was applied to that field within 1 min of collection by advancing the Hamilton syringe.

Macrocollection of extracellular message samples. Larger samples (0.5 ml) of medium containing extracellular message were collected from astrocytes grown in 24-well culture plates (2 cm^2 surface area). Before mechanical stimulation, the cells were washed three times with observation saline, with the final wash leaving 0.5 ml of saline in the well. Calcium waves were then mechanically stimulated at multiple sites by dropping glass microbeads (30–50 μm; Polysciences, Warrington, PA) into the well. As numerous beads landed on the astrocyte layer, calcium waves were mechanically stimulated simultaneously from the numerous landing points in the well. Four hundred microliters of saline (now containing released extracellular message) were collected 30 sec later. For most experiments, the material was bioassayed within 1 min of collection or frozen for subsequent ATP determination (below). In several experiments, the material was maintained at room temperature for 5–30 min; no loss of biological activity was detected during this time.

Treatment of extracellular message samples. Suramin (Sigma, St. Louis, MO) and pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS; Research Biochemicals, Natick, MA) were dissolved in observation saline at twice the final concentration and were added to the test well for 5 min. An equal volume of sample was then added to the well.

A stock solution of apyrase (300 U/ml; apyrase grade III; Sigma) was made immediately before use. Thirty microliters of the apyrase stock solution were added to 300 μl of sample (to give a final concentration of 27 U/ml) and incubated for 15 min at room temperature before testing. A similar treatment of observation saline with apyrase resulted in no detectable calcium response in astrocytes. Maintaining samples at room temperature for 15 min without apyrase did not result in any apparent reduction in biological activity.

Bioassay for extracellular messengers. Astrocytes in 24-well culture plates were loaded with fluo-3 AM as described above. The volume in an individual well was reduced to 250 μl. Image acquisition was begun. After three control frames, 250 μl of test solution was added to the well, and the image acquisition continued for an additional 45 sec. The number of cells responding to the test solution (i.e., at least one-half of the pixels for that cell reaching the criterion of $F/F_0 > 1.25$) was counted from this image sequence.

ATP measurement. ATP was measured using a luciferin/luciferase bioluminescence assay (Molecular Probes) and a luminometer (Monolight 1500; Analytical Luminescence Lab). Experimental samples and controls containing known concentrations of ATP were examined in a blinded manner and compared with an ATP standard curve. Each sample was run in triplicate.

RESULTS

An extracellular messenger for propagation of glial calcium waves

Two observations set the stage for this investigation by independently confirming, in cultures identical to those used throughout the bulk of these experiments, the existence of an extracellular message. Naturally occurring, physically isolated "islands" of astrocytes appear in cultures before the cultures reach confluency. Stimulation of a calcium wave on one such island lead to elevated calcium levels in other, adjacent islands, even over distances in excess of 50 μm (Fig. 1). Careful phase-contrast and fluorescence microscopic examination confirmed that there were no cellular contacts between these islands of astrocytes. This result was obtained even when a single isolated astrocyte was stimulated in sparsely seeded cultures (data not shown). This configuration is the natural analog of the experiments presented in our previous report (Hassinger et al., 1996). Because these

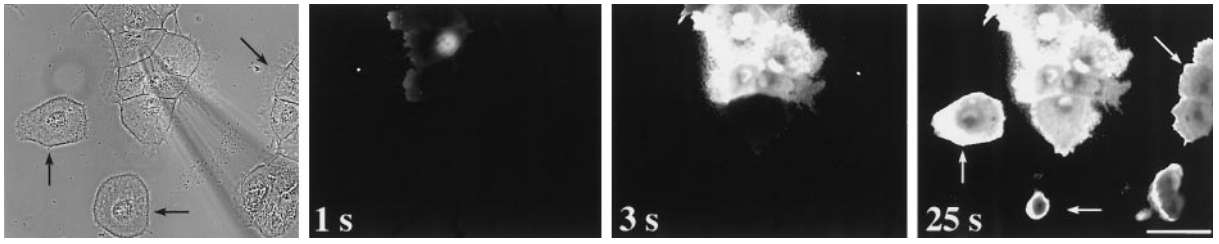

Figure 1. Communication between noncontacting islands of astrocytes. Stimulation of an astrocyte island (approximately six contiguous cells) results in a calcium wave passing throughout the stimulated island and in activation of astrocytes in the noncontacting islands (*arrows* in *left* and *right panels*). *Left*, A phase-contrast image of four noncontacting islands of astrocytes. One astrocyte in the *top* island was then electrically stimulated. *Middle*, *right*, F/F_0 ratio images that show progression of the calcium wave throughout the field at the times indicated. Scale bar, 50 μm.

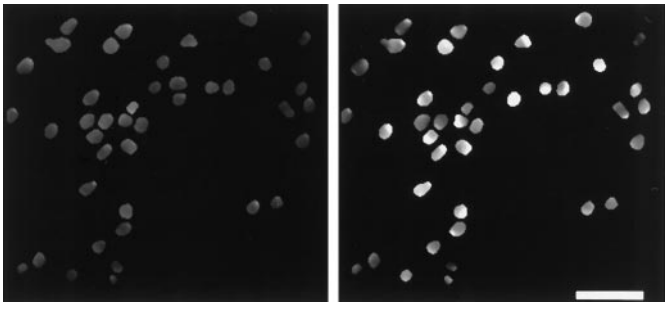

Figure 2. Human neutrophils respond to an extracellular message released during glial calcium waves. *Left,* Neutrophils were loaded with the calcium indicator fura-2 and seeded onto a astrocyte monolayer. *Right*, After electrical stimulation of a calcium wave in the astrocytes, calcium levels in the majority (but not all) of the neutrophils increased. Because the astrocytes were loaded with fluo-3, they did not appear in these images. The astrocytic fluo-3 fluorescence was used, in other regions of the culture, to confirm electrical stimulation efficacy before addition of the neutrophils. Scale bar, 50 μm.

islands had never been in physical contact with each other, there is no possibility of the existence of any residual cytoplasmic bridge, a potential, but extremely unlikely, explanation for the results of those previous experiments.

As a second approach, human neutrophils, which are known not to form gap junctions (Trosko and Ruch, 1998) except under very specific conditions of activation (Huang et al., 1993), were dropped onto an astrocytic culture as "mobile reporters" of released excitatory substances. A calcium wave was then electrically evoked in the astrocyte monolayer. The fura-2–loaded neutrophils responded to the astrocytic calcium wave with elevations in calcium (Fig. 2), with >50% of the neutrophils in the microscope showing calcium elevations ($n = 10$). Gently tapping the dish or gently adding medium to the dish demonstrated that the neutrophils had not adhered to the underlying astrocytes, indicating that no gap junctions had been formed during the 5 min period needed for these experiments. When astrocytes, acutely dissociated from culture flasks, were substituted for neutrophils in parallel experiments, they also displayed calcium responses to evoked calcium waves in the underlying glial monolayer (data not shown). The ability of mobile reporters to respond to calcium waves confirms the existence of an extracellular messenger. The fact that neutrophils responded to glial calcium waves has important pathological implications that will be discussed below and, as well, provided some guidance for our subsequent efforts to identify the active compound(s) used in extracellular communication.

Clearly, astrocytic calcium waves can propagate across acellular regions to other cells in the absence of any gap junction communication. These data reinforce the conclusion (Hassinger et al., 1996) that astrocytes are responding to an extracellular message released during calcium waves.

Extracellular message is present in medium after propagation of a calcium wave

Because the extracellular signal can cross acellular distances of >80 μm, it is probably released in high concentrations (relative to receptor sensitivity). We therefore determined whether one could collect samples of extracellular medium and assay for the presence of an excitatory substance that is released during calcium waves.

A procedure was developed to allow the collection of 0.1–0.2 μl of medium during a calcium wave. This medium could then be applied to unstimulated astrocytes in another region of the culture, to which the wave had not propagated, and thereby test for the presence of an excitatory substance. As seen in Figure 3, medium collected during a calcium wave consistently elevated calcium levels in previously unstimulated astrocytes ($n = 9$). Although the collected medium was normally applied to a test field within 1 min, in several experiments the medium could be retained in the collection pipet for at least 5 min without detectable loss of biological activity. Control medium (collected in the same manner from unstimulated astrocytes and applied to other unstimulated astrocytes) never had an effect on astrocytic intracellular calcium levels ($n = 10$).

Although the microcollection procedure clearly demonstrated that the extracellular message is released into the bathing medium during a calcium wave, it would not be likely to provide sufficient material from a single calcium wave, which was limited in extent, to identify the extracellular message. Therefore, we developed a procedure using mechanical stimulation of multiple sites throughout a culture to generate larger quantities of biologically active samples of extracellular message.

Mechanical stimulation of glia is commonly used in several laboratories to evoke calcium waves (Sanderson et al., 1990; Boitano et al., 1992; Charles, 1994; Naus et al., 1997; Wang et al., 1997). We were able to mechanically stimulate glia with the gravitational force of small (30–50 μm in diameter) glass beads dropped through ~3 mm of medium onto confluent astrocyte monolayers in single wells of a 24-well culture plate (Fig. 4). There were no detectable adverse affects on the astrocytes under the glass beads because (1) fluo-3 fluorescence intensity under the bead returned to normal, indicating that calcium levels returned to normal and that no dye leaked out of the cells during bead contact (i.e., membrane integrity was maintained), and (2) when local perfusion was used to move glass beads to different positions on the monolayer, the astrocytes under the original landing point

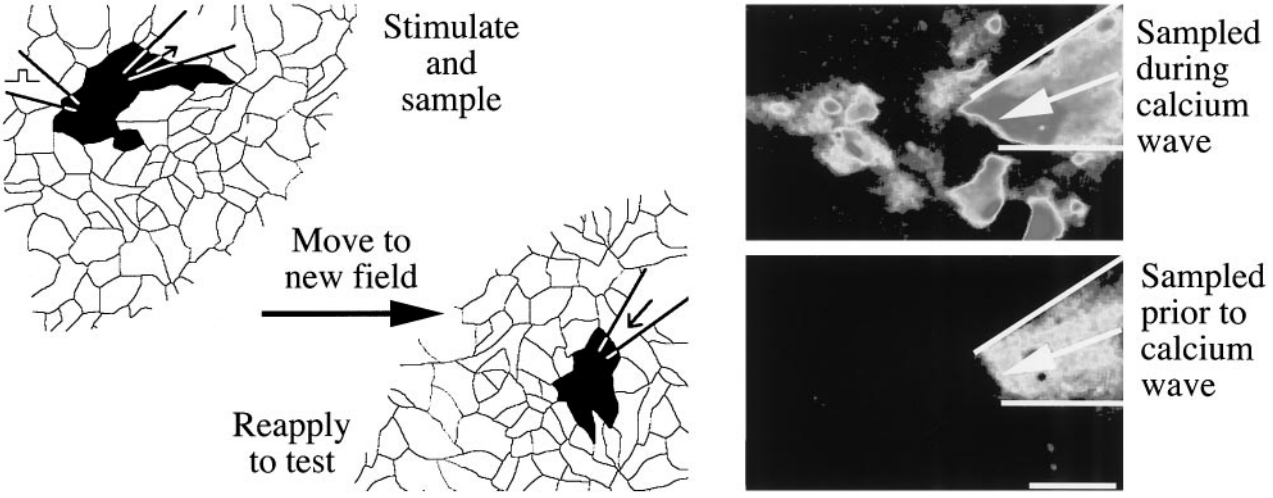

Figure 3. Collection of extracellular message during an evoked calcium wave. *Left*, A schematic representation of the experimental protocol. A patch pipet with a 3–5 μm opening was positioned 10 μm above the astrocyte surface. A calcium wave was electrically evoked using an extracellular stimulation electrode, placed several cells away from the collection pipet. As the wave, monitored in real time, passed under the collection pipet, negative pressure was applied to the pipet, collecting 0.1–0.2 μl of medium. The stage was then rapidly moved to bring a distant area of the culture into the microscope field. The collection pipet was then brought into proximity of the naive astrocytes in that field, and the collected medium was gently expelled onto those astrocytes (*arrow* in the pipet indicates the direction of flow). *Right*, The typical results of such an experiment. When the collected medium was gently applied to those astrocytes, a dramatic rise in calcium was observed (*top*). Control medium (collected before calcium wave stimulation) had no effect (*bottom*), demonstrating that the response to the stimulated-field medium was not caused by a pressure artifact. (As the medium was expelled from the pipet, the oil filling the collection system advanced to the pipet tip; this change in material filling the pipet tip accounts for the change in apparent fluorescence observed within the pipet tip.) The calcium wave during which the sample was collected is not shown. Scale bar, 50 μm.

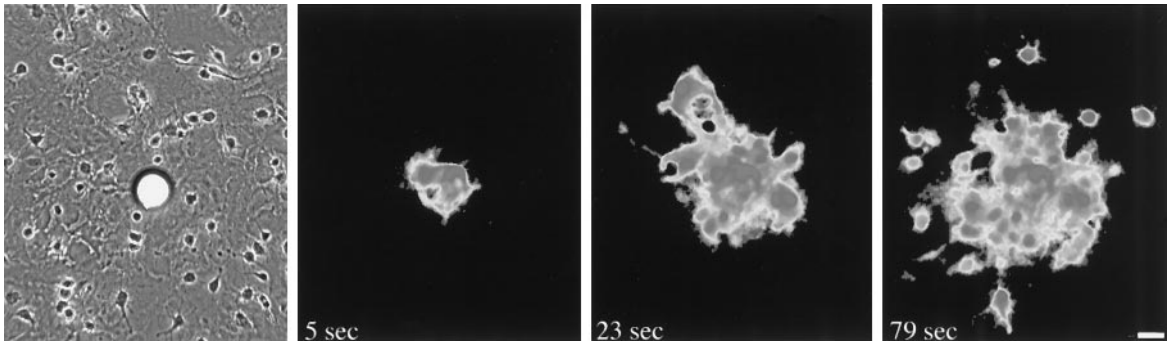

Figure 4. Mechanical stimulation of a calcium wave using glass microbeads. A small glass bead (30–50 μm), dropped through the medium onto a culture surface, initiates a calcium wave at the point where the bead lands. When thousands of beads are dropped throughout a single culture, thousands of such waves are simultaneously initiated and subsequently propagated throughout the culture. In this example, a single bead landed in the center of the field (phase-contrast image in the *left panel*, acquired after the fluorescence images), evoking the calcium wave seen in the *middle* and *right panels*. Scale bar, 50 μm.

appeared normal using both phase-contrast and fluorescence microscopy.

Astrocytes were simultaneously stimulated at multiple sites by dropping many glass beads (0.1 gm of total dry weight) throughout a single well of confluent astrocytes. After 30 sec, 0.5 ml of the original 0.6 ml of saline was removed and briefly centrifuged to pellet any beads that might have been collected with the sample. This material had significant levels of biological activity because addition of a sample aliquot to another well of the same 24-well culture plate (to a final 50% dilution) evoked significant calcium responses ($F/F_0 > 1.25$) in many of the astrocytes (Fig. 5). Medium samples taken from unstimulated cultures evoked no measurable calcium responses in test astrocytes. This bioassay procedure was used to identify the major active component in these samples.

The extracellular messenger exhibits purinergic properties

We initially tested the possibility that one component of the extracellular message was ATP for several reasons. (1) Our previous publication provided evidence that glutamate was not the primary compound used by glia for extracellular communication (Hassinger et al., 1996). (2) Neutrophils, which responded to the astrocytic calcium wave, respond to ATP (Kuroki et al., 1989; Merritt and Moores, 1991; Gasmi et al., 1997) but do not show calcium responses to many of the other neurotransmitter substances known to be released from astrocytes. (3) ATP is used as a diffusible extracellular messenger by other cell types (Osipchuk and Cahalan, 1992; Schlosser et al., 1996; Frame and de Feijter, 1997; Jørgensen et al., 1997). (4) Focal application of ATP can initiate glial calcium waves (van den Pol et al., 1992; Newman and

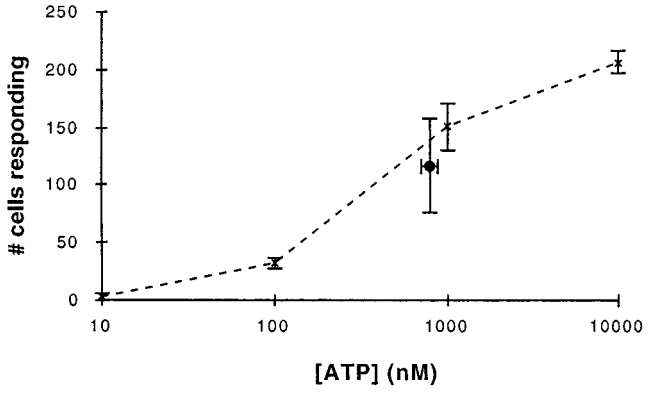

Figure 5. ATP dose–response curve and the comparison with experimentally collected extracellular message samples. ATP evokes calcium responses in astrocytes. The number of astrocytes responding to control medium containing known amounts of exogenous ATP was determined and plotted against ATP concentration (mean ± SEM; four separate cultures used for each data point). The *filled circle* represents the mean number of cells responding to the medium of three separate samples containing extracellular message. The concentration of ATP in each sample was measured using the luciferin/luciferase assay. This point fell remarkably close to the dose–response curve determined using medium containing exogenous ATP. Thus, ATP is present in extracellular message samples at concentrations sufficient to account for much of the biological activity measured in those samples.

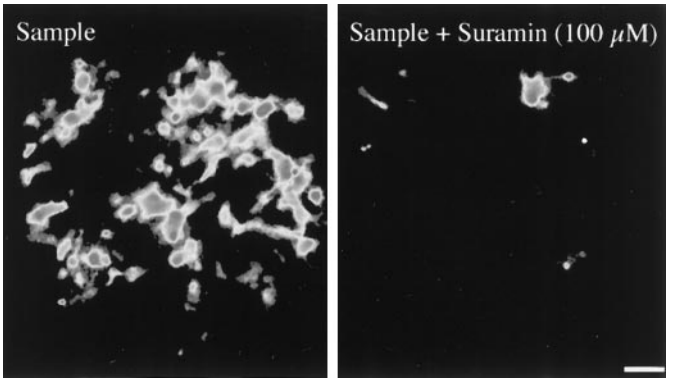

Figure 6. Purinergic antagonists block the biological activity of collected samples of extracellular message. *Left*, Samples of extracellular message evoke calcium responses in a majority of the astrocytes in the microscope field. *Right*, Addition of an aliquot of the same sample in the presence of suramin (100 μM final concentration) virtually eliminated the biological activity. (The images shown are integrated to display every astrocyte responding to the sample during the time course of the experiment and do not represent a single time point.) Scale bar, 100 μm.

Zahs, 1997). (5) Collected material maintains biological activity at room temperature for at least 30 min, suggesting that the biological activity is relatively chemically stable and effectively ruling out unstable compounds such as nitric oxide. (6) A variety of different purinergic receptor subtypes has been characterized (Dubyak and el Moatassim, 1993; North and Barnard, 1997), with evidence of P2X (Walz et al., 1994), P2U (Chen and Chen, 1996; King et al., 1996), and P2Y (Pearce and Langley, 1994; King et al., 1996) receptor subtypes expressed by astrocytes.

A bioassay was used in which an aliquot of an extracellular message sample, or saline containing ATP at a known concentration, was added to fluo-3–loaded astrocytes in 1 well of a 24-well plate. The calcium responses of the astrocytes were monitored for 45 sec after addition of the aliquot; the number of astrocytes that showed criterion changes were counted.

A standard dose–response curve of the number of astrocytes showing calcium responses versus ATP concentration showed the assay to be sensitive over more than two orders of magnitude of ATP concentration (Fig. 5). ADP was equally potent in evoking calcium responses in astrocytes. On the other hand, adenosine alone, which would result from the degradation of ATP by ectonucleotidases (Dubyak and el Moatassim, 1993), required 100-fold higher concentrations to evoke calcium responses in astrocytes. Furthermore, the calcium response to adenosine required 15–20 sec to develop, in contrast to the rapid (1–3 sec) response to ATP or ADP. Therefore, the response to ATP was likely caused by P2X or P2Y receptors and not by ATP degradation and subsequent activation of P1 receptors by adenosine.

Three different extracellular message samples evoked responses in an average of 117 ± 40 cells/field (mean ± SEM), similar to the activity of 1 μM ATP standards. By the use of the sensitive luciferase bioluminescence assay, ATP was detected in samples of extracellular message at a concentration of 780 ± 87 nM ($n = 3$) but not in control samples taken from unstimulated cultures (<20 nM). (The local concentration of ATP at the releasing astrocytes was likely to have been higher, because that released ATP would not have had time to equilibrate fully with the overlying 3 mm of medium.) These results supported our initial hypothesis that a primary extracellular messenger is ATP.

To test for the involvement of purinergic receptors in this excitatory response, extracellular message samples (0.5 ml) generated by glass bead stimulation were collected and split into two aliquots, one of which either was treated with apyrase or had purinergic antagonists added. Both aliquots were assayed on fluo-3–loaded test astrocytes in separate wells of a 24-well plate.

The general P2 antagonist suramin (100 μM) (Dubyak and el Moatassim, 1993) abolished the biological activity of the samples of extracellular message (Fig. 6). Samples of extracellular message evoked criterion F/F_0 changes in 63 ± 23 of the cells in the microscope field; addition of suramin reduced the response to 1 ± 1 cells ($n = 11$ separate cultures; $p < 0.01$). The more selective P2X/P2Y antagonist PPADS (Dubyak and el Moatassim, 1993; Ho et al., 1995) had the same effect; addition of PPADS (10 μM) reduced the number of responding cells from 75 ± 6 to 1 ± 1 ($n = 5$; $p < 0.05$). In both cases, the antagonist concentration was sufficient to block the excitatory effect of 1 μM ATP ($n = 4$; data not shown). Both results supported a preliminary identification of ATP as the active compound in samples of extracellular message.

Because of the inherent limitations of pharmacological antagonists, an entirely different approach was used as an additional test for the presence of ATP in extracellular message samples. Treatment of samples with the ATP-degrading enzyme apyrase (27 U for 15 min) reduced the biological activity from 102 ± 23 to 1 ± 1 cells ($n = 6$; $p < 0.005$). Similar treatment also eliminated the biological activity of 1 μM ATP standards ($n = 3$;

Calcium wave propagation is mediated by ATP

Taken together, the preceding experimental results demonstrate that ATP is released from stimulated astrocytes in sufficient amounts to evoke calcium responses in nearby astrocytes and that ATP is the major active component in the samples of extracellular message. Our final two sets of experiments examined directly the involvement of ATP in calcium wave propagation. To demonstrate an involvement of ATP in calcium wave propagation, it is necessary to show that (1) locally applied ATP can initiate a

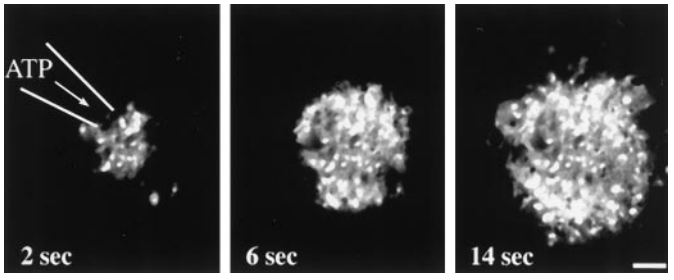

Figure 7. Local ATP application initiates astrocytic calcium waves. ATP (10 μM) was locally applied from a patch pipet (*arrow*) using a pressure pulse 20 msec in duration. *Left*, Astrocytes in the path of the applied ATP responded with an immediate calcium elevation. *Middle*, *right*, The resulting calcium wave is clearly seen. Scale bar, 100 μm.

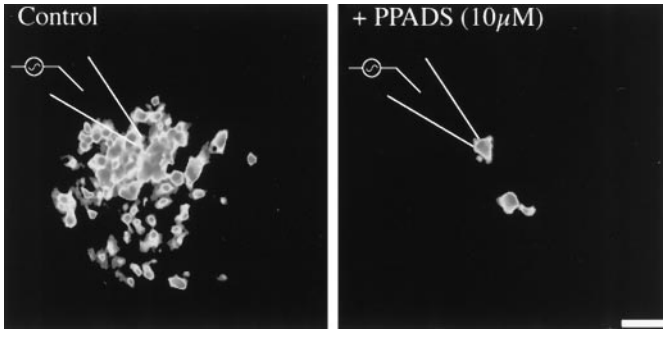

Figure 8. Purinergic antagonists block the propagation of an electrically evoked calcium wave. *Left*, When a single astrocyte was electrically stimulated, the evoked calcium wave propagated through many of the astrocytes in the field, normally involving 40 ± 7 cells. *Right*, Addition of *PPADS* (10 μM) to the observation saline virtually eliminated propagation of the calcium wave; only 6 ± 1 astrocytes show criterion calcium responses. Similar results were obtained using suramin (100 μM). The astrocytes participating in the presence of *PPADS* included the astrocyte(s) immediately adjacent to the stimulating electrode, which were likely to be stimulated directly. (The images shown are integrated to display every astrocyte responding to the sample during the time course of the experiment and do not represent a single time point.) Scale bar, 100 μm.

calcium wave and (2) propagation of an evoked calcium wave is prevented by blocking purinergic receptors.

Figure 7 shows the calcium wave resulting from local application of ATP. A 20 msec pressure pulse was applied to a patch pipet filled with saline containing 10 μM ATP. Calcium elevations were initially seen in the astrocytes immediately in the path of the ejected ATP (Fig. 7, *left*). The propagating calcium wave is seen in the *middle* and *right panels*. Wave initiation was obtained in >15 such experiments. ATP is therefore capable of initiating a calcium wave.

Focal electrical stimulation was used to determine whether blocking purinergic receptors also prevented propagation of a calcium wave. In control saline, focal electrical stimulation evoked waves consisting of 40 ± 7 participating cells (27 waves in seven separate experiments). The number of astrocytes was reduced to 5 ± 1 cells in the presence of 100 μM suramin (9 waves in three separate experiments; $p < 0.05$). PPADS (10 mM) reduced the number of participating astrocytes to 6 ± 1 cells (13 waves in four separate experiments; $p < 0.05$) (Fig. 8). In these experiments, the astrocytes participating in the presence of the antagonist must have included, but were not limited to, the astrocyte(s) immediately under the stimulating electrode that were stimulated directly.

DISCUSSION

Extracellular communication underlies calcium wave propagation

During calcium waves, mouse cortical astrocytes release substance(s) into the medium that evokes calcium responses in adjacent astrocytes, under the conditions of these experiments. Our previous work demonstrated that this extracellular communication pathway was sufficient for calcium wave propagation; calcium waves propagate across acellular regions in the absence of any possible gap junction communication. This extracellular communication system is also necessary for full calcium wave propagation in confluent cultures; movement of the extracellular medium strongly influences both the direction and the extent of calcium wave propagation. The mobile reporters used in the present study reinforce the conclusion that an excitatory substance(s) is released during calcium waves.

Despite substantial evidence of extracellular communication, the messenger had not been identified in previous studies. Several studies have demonstrated that glial activation of neuronal calcium transients involves glutamate (Parpura et al., 1994; Hassinger et al., 1995). However, our previous report provided evidence that glutamate is not the primary extracellular messenger for astrocytic calcium waves. The present study identifies the primary active component as ATP.

ATP is the primary extracellular messenger

The experiments reported here tested the hypothesis that ATP is involved in extracellular communication between astrocytes. The effects of the extracellular message have been mimicked by application of purinergic agonists and masked by purinergic antagonists and by specific degradative enzyme treatment. ATP can be measured in medium samples after mechanical stimulation of astrocyte cultures. Finally, purinergic antagonists almost completely abolish the propagation of stimulated calcium waves. Taken together, these results strongly support the conclusion that ATP is the primary effective compound of the extracellular communication system.

Clearly, stimulation results in the release of ATP, which can evoke calcium responses in astrocytes. It is important to consider whether such release might in any way be an artifact of the type of stimulation used. In the experiments included in this study, neither mechanical nor electrical stimulation produced any visible damage to the stimulated astrocytes. Fluorescence intensity levels in the stimulated astrocytes returned to normal after stimulation, suggesting that calcium levels had also returned to original levels and that no indicator was lost because of damage. Furthermore, individual astrocytes could be stimulated many times (at appropriate intervals) to evoke quite similar calcium waves. These observations suggest that the stimulation used in this study was not evoking ATP release by membrane damage. In addition, a previous investigation (Queiroz et al., 1997) had found that ATP release can be evoked by receptor activation, a form of stimulation quite like that expected *in vivo*. Although one must always be critical of artificial forms of stimulation, these observations, taken together, relieve much of the concern about the physiological relevance of stimulation-evoked ATP release from astrocytes.

Our previous study (Hassinger et al., 1996) provided evidence supporting the idea that cells along the wave path also released extracellular messenger during the calcium wave. Nonetheless,

the relative contribution of ATP released by the stimulated cell(s) and by cells along the path remains to be determined. A theoretical approach to this issue is illuminating. A model for the generation of calcium waves involving the point source release of a diffusible extracellular messenger from a single, stimulated astrocyte is equivalent, in form, to the model proposed by Sneyd et al. (1994) in which a point source elevation of an intracellular messenger, e.g., IP_3, passes through gap junctions into other cells. The most notable feature of a point source model is that the calcium wave would be of finite extent. An alternative model, supported by our previous work, includes the release of extracellular messenger not only by the stimulated cell but also, in a sequential manner, by cells along the path of the calcium wave. Thus, the response is regenerative. In principle, if each participating astrocyte rapidly released sufficient amounts of ATP during a calcium wave, an essentially infinitely propagating calcium wave would result.

We do not see infinitely propagating calcium waves but rather waves that propagate on the order of 20 cell diameters. This finite wave extent could be explained by a point source model. Alternatively, if not all astrocytes respond to or release ATP, calcium waves could also be limited in extent. There is reason to expect that not all astrocytes will show identical responses to ATP. Astrocytes express different purinergic receptor subtypes (Pearce and Langley, 1994; King et al., 1996), with subpopulations of astrocytes expressing specific receptor subtypes (Ho et al., 1995). In addition, our dose–response data (Fig. 5) show that some astrocytes are more sensitive (in terms of their calcium responses) to ATP than are others.

An additional piece of evidence against the single point source release of ATP can be derived from a quantitative assessment of the predicted diffusion of the extracellular messenger from a point source (Crank, 1975) based on the measured diffusion coefficient for ATP (Hazel and Sidell, 1987). Assuming an astrocyte has a cytoplasmic ATP concentration of 5 mM [a high estimate (Dubyak and el Moatassim, 1993)] and releases 10% of that ATP in response to stimulation (again, likely a very high estimate), we can determine the theoretical distance at which diffusing ATP would remain above threshold (≥100 nM) for the generation of calcium responses in astrocytes. Such suprathreshold levels would not be found at distances >100 μm from the stimulated cell. In reality, however, calcium waves normally propagate an average of 360 μm ($n = 20$ in the present experiments) (see, e.g., Fig. 8). Furthermore, in the extreme modeled case of 100% ATP release, threshold levels of ATP still would only reach a distance of 220 μm. Clearly, then, this quantitative analysis provides spatial arguments against calcium waves being attributable entirely to ATP release from a single point source.

Temporal aspects of the model also argue against a point source model. ATP diffusion from a point source as described above would, in theory, result in suprathreshold concentrations progressing at an average rate of ~40 μm/sec. In reality, however, the evoked calcium wave travels at a nearly constant rate of 10 μm/sec (Hassinger et al., 1996). Taken together, the temporal and spatial aspects of a point source model seem incompatible with our observed calcium waves. We conclude that calcium wave propagation by an extracellular messenger is a different process that is likely to include sequential release of ATP by cells along the path of the wave. The issue remains as to why waves do not display longer propagation distances; perhaps the system is only moderately regenerative under the conditions of our experiments.

Our new finding of the involvement of ATP in glial calcium wave propagation is, in fact, supported by previous literature. Individual components of a purinergic extracellular signaling system have been demonstrated in diverse systems and by different investigators. Cultured astrocytes can release ATP in response to glutamatergic receptor stimulation (Queiroz et al., 1997) and respond to ATP with calcium elevations both *in vitro* (McCarthy and Salm, 1991; van den Pol et al., 1992; Salter and Hicks, 1994; Centemeri et al., 1997) and *in situ* (Kriegler and Chiu, 1993; Bernstein et al., 1996; Newman and Zahs, 1997). Additionally, suramin, which blocks purinergic receptors [although it has other biological effects (Chahdi et al., 1998)], blocks glial calcium waves (Guan et al., 1997; Zanotti and Charles, 1997). Finally, ATP has been shown to mediate extracellular communication in several different cell types including mast cells (Osipchuk and Cahalan, 1992), osteoblasts (Jørgensen et al., 1997), and hepatocytes (Schlosser et al., 1996).

These results, together with our previous publication, strongly question the older view that gap junction coupling wholly mediates glial calcium wave propagation, a conclusion based primarily on the ability of classical "gap junction blockers" to block glial calcium wave propagation (Charles et al., 1992; Finkbeiner, 1992; Nedergaard, 1994; Venance et al., 1995, 1997). Two more recent studies now support the idea that gap junction communication is not required for glial calcium wave propagation. Astrocytes cultured from connexin-43 (the primary connexin expressed by astrocytes) knock-out mice have very limited dye and electrical coupling; calcium waves still propagate, although to a reduced extent (Naus et al., 1997). Guan et al. (1997) showed that anandamide and oleamide abolish dye and electrical coupling without affecting the calcium wave propagation; however, 18β-glycyrrhetinic acid and heptanol, commonly used to block gap junctions, block both gap junction coupling and calcium wave propagation (Guan et al., 1997). They suggested that 18β-glycyrrhetinic acid and heptanol, as well as other gap junction blockers used in previous reports, might also block an extracellular communication pathway.

There are multiple mechanisms involved in calcium wave propagation

Although the present study demonstrates a significant role for ATP in cortical glial calcium wave propagation, all extracellular communication does not need to involve purinergic receptors. Purinergic antagonists did not completely block responses to extracellular message samples. Apyrase treatment did not completely eliminate the excitatory activity in those samples. Finally, not every mobile reporter astrocyte showed a calcium response during a calcium wave in the underlying monolayer.

The list of compounds evoking calcium rises in astrocytes is quite large, and astrocytes show considerable diversity in phenotype. Individual astrocytes can respond to different subsets of neurotransmitters both *in vivo* (Bernstein et al., 1996) and in culture (McCarthy and Salm, 1991). The sensitivity of astrocytes seems to be developmentally regulated (Bernstein et al., 1996) and even altered by specific pathological conditions (Enkvist et al., 1996; Gottlieb and Matute, 1997; McKeon et al., 1997). One or more of these other compounds could be used as an extracellular messenger, between specific astrocytes under specific conditions. Thus, it is tempting to speculate that the connectivity of glial circuits can show plasticity, much as is seen in neuronal circuits.

In addition, astrocytes from different brain regions display different degrees of gap junction coupling (Lee et al., 1994). This diversity could extend to the mechanisms underlying calcium

wave propagation. Calcium waves are blocked by anandamide in striatal astrocytes (Venance et al., 1995, 1997) but not in cortical astrocytes (Guan et al., 1997). Apyrase treatment, which should block purinergic communication by degrading ATP, has no effect on calcium wave propagation through striatal astrocytes (Venance et al., 1997). On the other hand, the purinergic antagonist suramin blocks calcium wave propagation in both rat (Guan et al., 1997) and mouse cortical astrocytes (this report) and blocks the propagation of spontaneously occurring calcium waves in astrocyte cultures after exposure to low external calcium medium (Zanotti and Charles, 1997). Striatal and cortical astrocytes seem to use different mechanisms for calcium wave propagation, with striatal astrocytes using gap junction communication as the primary mechanism and cortical astrocytes primarily using extracellular communication.

The present findings, therefore, might tie together a large number of earlier publications that individually demonstrated that numerous compounds are released from and/or responded to by astrocytes, but with function(s) largely a matter of speculation or incomplete understanding. Some of these other compounds might be involved in calcium wave propagation, with selective receptor expression providing the possibility for selective interglial communication. Furthermore, astrocytes in some brain regions might use gap junctions and extracellular signaling, to different extents, as parallel communication systems. Such findings would significantly expand the potential for specific glial circuitry, adding to the richness of possible forms of specific interaction that underlie the complexity and thereby power of the nervous system.

Interglial communication has implications for both normal and pathophysiological functioning of the nervous system

All of these observations suggest that interastrocyte communication might be much more sophisticated than had been assumed previously. The existence of glial circuits would have marked impact on the way in which we view nervous system function. Glial circuits and neuronal circuits seem to provide parallel communication pathways within the nervous system. The interaction of such glial and neuronal circuits (Dani et al., 1992; Nedergaard, 1994; Parpura et al., 1994; Hassinger et al., 1996) could provide opportunities for significantly altered computation capabilities and functional plasticity within the brain.

There are several important issues that should be considered when trying to relate the findings of the present investigation to events *in situ*. Foremost among these is the fact that astrocytes *in situ* are in close approximation to neurons. Accordingly, ATP released from astrocytes could well directly influence neurons with appropriate purinergic receptors. In addition, it is reasonable to consider that not all astrocytes may in fact have ATP receptors or be able to release ATP as they are under the conditions in the present experiments. In this regard, it is interesting to note that one study has reported that purinergic responses are infrequently seen in astrocytes acutely dissociated from early postnatal rats (Kimelberg et al., 1997). We have confirmed these results but have also observed, in preliminary experiments, that astrocytes acutely dissociated from adult mouse cortex are fivefold more likely to respond to ATP. One could therefore envision that the specific mechanisms underlying calcium wave propagation could change during development. Furthermore, it is possible that both purinergic receptors and ATP release capabilities might exist in a majority of astrocytes in some brain regions and, alternatively, receptors and release capabilities might be found in only selected astrocytes in other regions. Those astrocytes could be members of a more complex circuit relying on multiple transmitters or, perhaps, a combination of extracellular- and gap junction-mediated propagation. Whatever the actual case, it seems clear to us that the situation described in the culture system used in the present experiments is likely to represent only one of the many ways in which release of an extracellular messenger from astrocytes can facilitate both propagation of calcium waves and interactions with their companion neurons.

Our results also might provide insights into pathological situations. Neutrophils and other blood cells are found within the CNS after stroke and various types of traumatic head injury (Witte and Stoll, 1997). A significant number of neurons die during a period of secondary cell death, a time during which numerous neutrophils have invaded the stroke site (Witte and Stoll, 1997). Neutrophils respond to ATP with elevated calcium levels, a primary trigger for neutrophil degranulation (Walker and Ward, 1992). ATP-evoked degranulation could result in a significant increase in local cell death. In addition, extracellular calcium levels plummet during and remain low immediately after global ischemia. Because exposure of mouse cortical glial cultures to low calcium media results in the generation of spontaneous calcium waves (Zanotti and Charles, 1997), glial activity could be increased above normal levels, resulting in even more ATP release. Glial calcium waves could, therefore, exacerbate the deleterious effects of stroke. Alternatively, adenosine, a breakdown product of ATP, might play a neuroprotective role after global ischemia (Rudolphi et al., 1992; Heurteaux et al., 1995; Schubert et al., 1997), suggesting that glial calcium waves might reduce CNS damage. In either case, glial–neutrophil communication could provide a pivotal target for therapeutic reduction of the extent of nervous system pathology after stroke.

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