THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 2, Issue of January 10, pp. 1354–1362, 2003

Printed in U.S.A.

Storage and Release of ATP from Astrocytes in Culture*

Received for publication, September 16, 2002, and in revised form, October 22, 2002
Published, JBC Papers in Press, October 31, 2002, DOI 10.1074/jbc.M209454200

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ATP is released from astrocytes and is involved in the propagation of calcium waves among them. Neuronal ATP secretion is quantal and calcium-dependent, but it has been suggested that ATP release from astrocytes may not be vesicular. Here we report that, besides the described basal ATP release facilitated by exposure to calcium-free medium, astrocytes release purine under conditions of elevated calcium. The evoked release was not affected by the gap-junction blockers anandamide and flufenamic acid, thus excluding purine efflux through connexin hemichannels. Sucrose-gradient analysis revealed that a fraction of ATP is stored in secretory granules, where it is accumulated down an electrochemical proton gradient sensitive to the v-ATPase inhibitor bafilomycin A_1. ATP release was partially sensitive to tetanus neurotoxin, whereas glutamate release from the same intoxicated astrocytes was almost completely impaired. Finally, the activation of metabotropic glutamate receptors, which strongly evokes glutamate release, was only slightly effective in promoting purine secretion. These data indicate that astrocytes concentrate ATP in granules and may release it via a regulated secretion pathway. They also suggest that ATP-storing vesicles may be distinct from glutamate-containing vesicles, thus opening up the possibility that their exocytosis is regulated differently.

Astrocytes propagate long-range calcium signals to neighboring cells and affect the activity of neurons by evoking calcium transients (1–6) and modulating neurotransmission (7, 8). It was first believed that calcium waves are propagated by the diffusion of intracellular messengers, such as Ca^{2+} and inositol 1,4,5-trisphosphate, across gap junctions (see Ref. 9, and Refs. therein). It has more recently become clear that calcium propagation may occur by means of an extracellular pathway because it also takes place among physically separated astrocytes (3, 10) or when gap-junction-mediated coupling is pharmacologically impaired (11, 12).

Several lines of evidence now suggest that ATP is the major extracellular messenger for inter-astrocyte calcium-mediated communication (10, 13, 14). First, ATP is released from astrocytes during calcium wave propagation (10, 14). Second, the propagation can be reduced or abolished by purinergic antagonists (10, 11, 13, 15, 16) or ATP-degrading enzymes (10, 12, 13). Finally, ATP mediates calcium-based intercellular communications between astrocytes and other cell elements such as meningeal cells (17), Muller cells (18), and microglia (19). These findings have highlighted the importance of ATP in cross-talk among astrocytes and between them and other cell types in the central nervous system.

The mechanisms of ATP release by glial cells has been actively investigated (9, 13, 14, 20–22). A recent study has reported a connexin hemichannel-mediated ATP release from astrocytes (21). Accordingly, in glioma cells, connexin expression potentiates both the resting and the stimulated ATP release (13). However, the possibility of a vesicular component of ATP release has never been investigated in astrocytes. ATP is known to be packaged in vesicles, being co-stored with acetylcholine in central and peripheral nerves, and with noradrenaline in the vesicles of sympathetic nerve terminals and in the related granules from chromaffin cells (23). Furthermore, evidence has been recently reported of vesicular ATP release from endothelial cells (24). The existence of typical secretory granules undergoing regulated secretion in astrocytes has recently been defined (25), and proteins of the synaptic vesicle fusion machinery have been identified in glial cells (26–28).

Because these findings could be consistent with vesicular storage and regulated purine secretion from astrocytes, we investigated the mechanisms of ATP storage and release from primary cultures of hippocampal astrocytes.

EXPERIMENTAL PROCEDURES

Materials—Antibodies against rat SgII[1] were raised in rabbits, purified by affinity chromatography, and characterized as previously described (25). The monoclonal antibodies against GFAP came from Roche Molecular Biochemicals; the polyclonal antibodies against colony-stimulating factor-1 receptor from Santa Cruz Biotechnology (Santa Cruz, CA); and the polyclonal antibodies against synaptobrevin/VAMPII were from Synaptic System GmbH (Gottingen, Germany). Ribophorin and complex 3 were kindly provided by Prof D. Borgese (Milan, Italy). The secondary antibodies conjugated to fluorescein isothiocyanate, Texas Red, 10 nm gold particles, and peroxidase were obtained from Jackson Immunoresearch Laboratories (West Grove, PA); APV, CNQX, MCPG,

* This work was supported by grants from the European Commission (QLGR3-CT-2000-01343, Coordinator Eric Scarfone), MURST-PRIN 2000; from Telethon -1042-, from HFSPO RGY0027/2001-B and Azienda Spaziale Italiana (I/R/149/00) (to M. M.) and from the Consiglio Nazionale delle Ricerche (Target Project on Biotechnology) (to P. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: SgII, secretogranin II; PMA, phorbol 12-myristate 13-acetate; KRH, Krebs-Ringer-Hepes; APV, 2-amino-5-phosphonovaleric acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; MCPG, α-methyl-4-carboxyphenilglycine; GFAP, glial fibrillar acidic protein; t-ACPD, 1-aminocyclopentane-*trans*-1,3-olicarboxylic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; PPADS, pyridoxalphosphate-6-azophenyl-2,4-disulfonic acid; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid tetrakis (acetoxymethyl ester).

t-ACPD, AMPA, and bafilomycin A_1 were from Tocris Neuramin (Bristol, UK); quinacrine dihydrochloride, bradykinin, glutamate, ATP, PPADS, PMA, anandamide, apyrase (grade II) and flufenamic acid, BAPTA/AM were from Sigma. The ATP assay kit came from Molecular Probes Europe (Leiden, NL) and the lactate dehydrogenase kit from Sigma (Milano, Italy).

Cell Cultures—Hippocampal mixed-glia cultures from embryonic rat pups (E18) were obtained using previously described methods (25). Briefly, after dissection, the hippocampi were dissociated by treatment with trypsin (0.25% for 10 min at 37 °C) followed by fragmentation with a fire-polished Pasteur pipette. The dissociated cells were plated onto glass coverslips at a density of 0.5×10^6 cells/ml, and the cultures were grown in minimum essential medium (Invitrogen) supplemented with 20% fetal bovine serum (Euroclone Ltd, UK) and glucose at a final concentration of 5.5 g/l (glial medium). To obtain a pure astrocyte monolayer, any microglia cells were harvested by shaking 3-week-old cultures. The primary hippocampal neuron cultures were prepared from E18 embryos as previously described (29).

Immunocytochemistry—The cultures were fixed for 25 min at room temperature with 4% paraformaldehyde in 0.12 M phosphate buffer containing 0.12 M sucrose. The fixed cells were detergent-permeabilized and labeled with primary antibodies followed by fluorochrome-conjugated secondary antibodies. The coverslips were mounted in 70% glycerol in phosphate buffer containing 1 mg/ml phenylendiamine. The images were acquired using a BioRad MRC-1024 confocal microscope equipped with LaserSharp 3.2 software. Electron microscopy was performed as previously described (25). Quinacrine staining was performed by incubating living cultures for 30 min at 37 °C with Krebs-Ringer-Hepes (KRH: 125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2 mM $CaCl_2$, 6 mM glucose, 25 mM Hepes/NaOH, pH 7.4) containing 5×10^{-7} M quinacrine dihydrochloride. Quinacrine-fluorescent living astrocytes were examined with a Zeiss microscope equipped with epifluorescence and photographated using a TMAX 400 (Eastman Kodak Co.).

Subcellular Fractionation and Immunoblotting—After being grown on Petri dishes until near confluence, the astrocytes were scraped, washed, and resuspended 1:4 in homogenization buffer (10 mM Hepes-KOH, pH 7.4, 250 mM sucrose, 1 mM Mg acetate, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 10 μg/ml aprotinin). The cells were homogenized using a cell cracker (European Molecular Biology Laboratory, Heidelberg, Germany) and centrifuged at $1,000 \times g$ for 10 min to prepare the post-nuclear supernatant. This supernatant was loaded onto a 0.4–1.8 M sucrose gradient and spun in a 41 SW rotor (Beckman Instruments, Inc., Palo Alto, CA) at 25,000 rpm for 18 h. Fractions (1 ml) were collected and analyzed by SDS-PAGE followed by Western blotting as previously described (25). Briefly, after electrophoresis, the proteins were transferred to nitrocellulose filters which, after being incubated in blocking buffer (5% milk, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl), were labeled with primary antibodies followed by the appropriate secondary antibodies conjugated to peroxidase diluted in blocking buffer containing 0.1–0.3% Tween 20. After extensive washing, the immunodecoration pattern was revealed using an enhanced chemiluminescence system (SuperSignal from Pierce, Rockford, IL) following the manufacturer' s protocol.

FURA-2 Videomicroscopy—The cultured cells were loaded for 60–90 min at 37 °C with 5 μM FURA-2 pentacetoxy-methylester in KRH, washed in the same solution, and transferred to the recording chamber of an inverted microscope (Axiovert 100; Zeiss, Oberkochen, Germany) equipped with a calcium imaging unit. For the assays, a modified CAM-230 dual-wavelength microfluorimeter (Jasco, Tokyo, Japan) was used as a light source. The experiments were performed using an Axon Imaging Workbench 2.2 equipped with a PCO SuperVGA SensiCam (Axon Instruments, Foster City, CA). The ratio values in discrete areas of interest were calculated from sequences of images to obtain temporal analyses. The images were acquired at 1–3 340/380 ratios/s. The experiments were performed in a static bath at room temperature (24–25 °C). The increases in calcium were quantified by measuring the peak and/or area of the response.

Glutamate Measurements—The biological assay for glutamate detection was performed as previously described (29). Specifically, monolayers of astrocyte cultures in 60-mm Petri dishes were kept in 1 ml of KRH in the absence and then in the presence of PMA or t-ACPD for 10–30 min at 37 °C. Neuronal cultures loaded with FURA-2 were then exposed to the different aliquots of KRH. Immediately before challenging, the aliquot collected from unstimulated astrocytes was supplemented with the stimuli. To verify that the biological activity of the conditioned medium was caused by accumulated glutamate, a subset of recordings were made in the presence of glutamate receptor antagonists, APV and CNQX (29, 30). Although embryonic hippocampal neurons lack t-ACPD receptors coupled to calcium mobilization,[2] the specific antagonist MCPG was always added to the conditioned medium upon t-ACPD stimulation. To test the tetanus neurotoxin (TeNT) sensitivity of glutamate release, the same astrocyte monolayer was challenged with PMA before and after 20-h incubation with the neurotoxin. Collected aliquots were frozen and then tested on the same FURA-2-loaded neurons. The endogenous glutamate concentration in the conditioned medium was determined by HPLC analysis coupled with fluorimetric detection as previously described (30).

ATP Measurements—Off-site ATP bioassay aliquots of KRH (1 ml), conditioned as described above for glutamate detection, were split into two parts before testing on FURA-2-loaded astrocytes. One part was pretreated with apyrase (30 units/ml) for 15 min before testing. Before being exposed to ATP sensor cells, each aliquot was supplemented with a mixture of glutamate antagonists (APV 100 μM, CNQX 20 μM, MCPG 1 mM) and the appropriate stimulus when conditioned under control conditions. KRH conditioned under mechanical stimulation was collected from astrocyte monolayers shaken for 5 min on an orbital shaker (Stuart Scientific, UK). The same aliquots were tested for lactate dehydrogenase activity following the manufacturer's protocol.

Bioluminescence Assay—ATP levels in the superfusates of pure astrocyte monolayers were measured using a luciferin/luciferase assay (Molecular Probes, Leiden, NL) and a luminometer (Lumat, Berthold, LB9501) according to the manufacturer's instructions. 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. ATP was detected on subcellular fractions by means of the same assay of equal aliquots of sucrose fractions that were boiled for 5 min before being frozen.

RESULTS

Biological Assays of ATP Release from Astrocytes—To study the mechanisms of ATP release from hippocampal astrocytes, both "on line" (Fig. 1*A*) and "off-site" (Fig. 1*D*) biological assays were performed. The first method is based on the finding that microglia co-cultured with astrocytes may act as ATP reporter cells by selectively responding to the ATP released from adjacent astrocytes as $[Ca^{2+}]_i$ increases (19). In this assay, FURA-2-loaded astrocyte-microglia co-cultures (Fig. 1*A*) were digitally imaged in the presence of glutamate receptor antagonists (100 μM APV and 20 μM CNQX) to exclude the possible contributions of released glutamate or D-serine. A gentle touch of the astrocyte with a glass pipette (a widely used stimulus for ATP secretion: 10, 12, 13) (Fig. 1, *A* and *B*) generated an increase in $[Ca^{2+}]_i$ in the stimulated cell, followed by a delayed $[Ca^{2+}]_i$ response in neighboring astrocytes and microglial cells. Despite the efficient propagation of the calcium signal among astrocytes (19), the microglia $[Ca^{2+}]_i$ responses were completely blocked or substantially inhibited when mechanically stimulated in the presence of the nonselective purinergic antagonist PPADS (50 μM) or the ATP-degrading enzyme apyrase (30 units/ml) (Fig. 1*C*). No significant changes in mean astrocyte calcium responses were recorded in the presence of PPADS or apyrase (percent changes in $\Delta 340/380$ fluorescence ratio: PPADS, 74.5 ± 7.8, $n = 5$, $p = 0.14$; apyrase, 115.68 ± 21.5, $n = 6$, $p = 0.43$, data normalized to controls). These data indicate that ATP is the extracellular messenger responsible for microglial $[Ca^{2+}]_i$ responses. Furthermore, a significant delay in the residual response was observed in the presence of the same blockers (a $514 \pm 42\%$ increase in the time to peak response in the presence of PPADS, and $420 \pm 35\%$ in the presence of apyrase, as compared with controls). Similar results were obtained when the mixed cultures were stimulated with 1 μM bradykinin (Fig. 1*C*), which selectively increases $[Ca^{2+}]_i$ in astrocytes (19).

The alternative bioassay for the study of ATP secretion was based on off-site measurements of released purine. Superfusates, conditioned by differently treated pure astrocyte monolayers, were added to FURA-2-loaded astrocytes as ATP sensor

[2] C. Verderio and M. Matteoli, unpublished data.

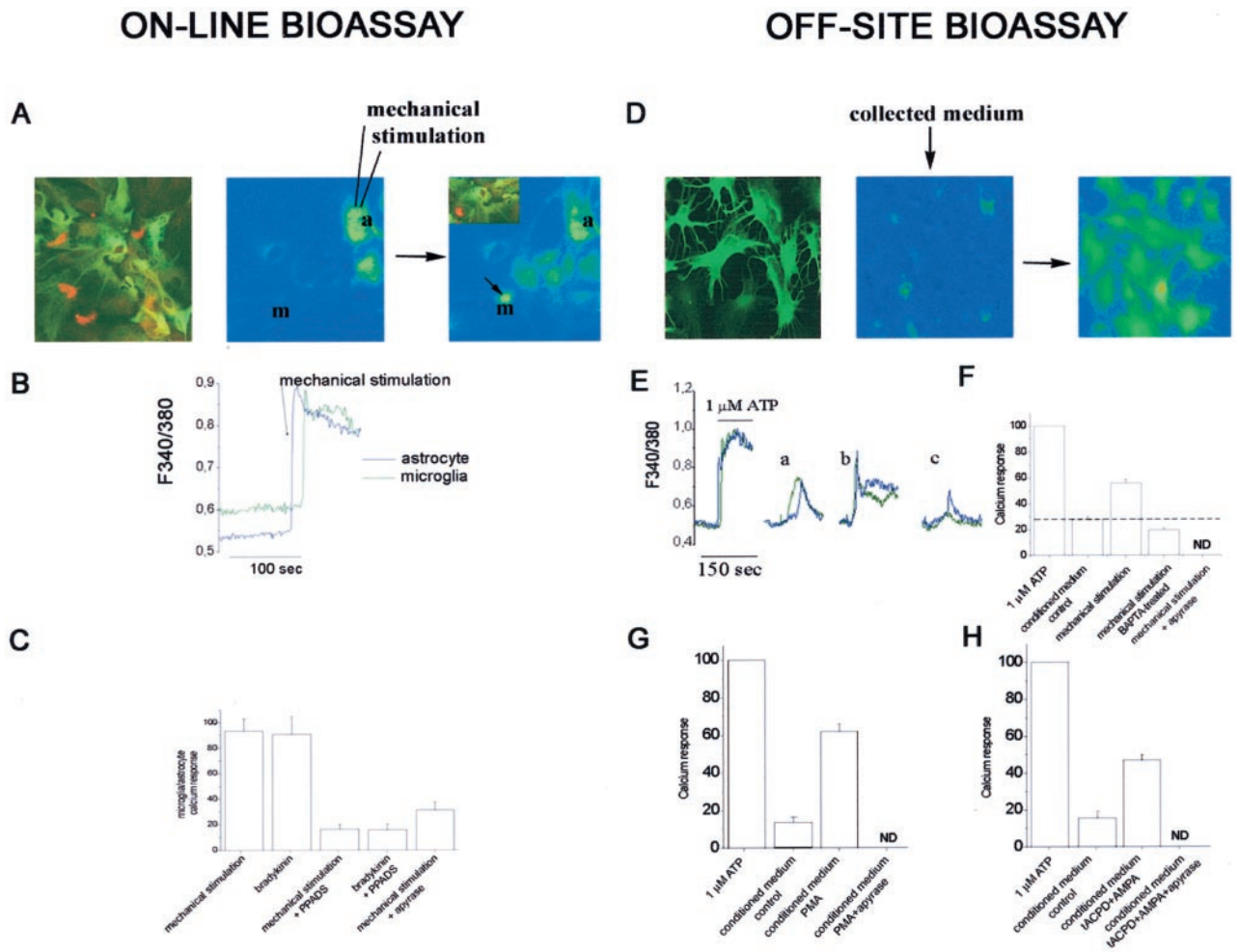

FIG. 1. **Calcium-dependent ATP secretion.** *A*, schematic representation of the on-line biological assay for ATP detection. *Left panel*, co-culture of astrocytes and microglial cells double stained for the astrocytic marker GFAP (*green*) and the microglial marker colony-stimulating factor-1 receptor (*red*). *Middle* and *right panels*, pseudocolor images of FURA-2-loaded mixed glial cultures showing the propagation of a mechanically induced calcium wave from astrocytes to a microglial cell. *Middle panel*, peak $[Ca^{2+}]_i$ response in the mechanical stimulated astrocyte (indicated by *a*); *right panel*: peak $[Ca^{2+}]_i$ rise in a microglial cell present in the field (indicated by *m*). *Inset*, double labeling of the same field with antibodies against GFAP (*green*) and colony-stimulating factor receptor (*red*). *B*, temporal plot of $[Ca^{2+}]_i$ changes recorded from a mechanically stimulated astrocyte (*blue trace*) and an adjacent microglia (*green trace*). Note the delay of the onset of the $[Ca^{2+}]_i$ response in the microglial cell. *C*, histograms showing the mean peak ± S.E. of the $[Ca^{2+}]_i$ response in microglia normalized to the peaks $[Ca^{2+}]_i$ increases in mechanically or bradykinin-stimulated astrocytes, under control conditions or in the presence of PPADS or apyrase (mechanical stimulation = 93.3 ± 9.3; $n = 14$; mechanical stimulation + PPDAS: 16 ± 3.4; $n = 8$; mechanical stimulation + apyrase: 31.25 ± 6; $n = 13$; bradykinin = 90.6 ± 13.8; $n = 12$; bradykinin + PPADS 15.75 ± 4.46; $n = 9$). *D*, schematic representation of the off-line biological assay for ATP detection. Medium conditioned by a pure astrocyte monolayer (*left panel*, staining for GFAP) induces a $[Ca^{2+}]_i$ response in FURA-2-loaded astrocytes as ATP sensor cells: pseudocolor images taken before (*middle panel*) and 30 s after (*right panel*) the addition of the conditioned medium. *E*, temporal plot of $[Ca^{2+}]_i$ increases recorded from two distinct FURA-2-loaded astrocytes after the addition of exogenous ATP and medium conditioned by the same astrocyte monolayer under static conditions (*a*), mechanical stimulation (*b*), and mechanical stimulation after BAPTA treatment (*c*). *F*, *G*, and *H*, quantitative analysis of ATP levels in medium conditioned by a pure astrocyte monolayer under control conditions and upon mechanical stimulation (*F*), or treatment with PMA (*G*) or t-ACPD and AMPA (*H*). The values represent percent changes (± S.E.) normalized to the $[Ca^{2+}]_i$ responses induced by the addition of exogenous 1 μM ATP. Note that, in all three cases, the conditioned medium pretreated with apyrase (30 units/ml) failed to evoke a $[Ca^{2+}]_i$ response. *F*, percent changes in response: 27 ± 2.6 under static conditions, 55.99 ± 2.7 under mechanical stimulation, 19 ± 1.8 after BAPTA treatment; $n = 28$ $p < 0.01$. *G*, percent changes in response: 18 ± 2.5 under control conditions, 62 ± 4 after PMA treatment. *H*, percent changes in response: 18.2 ± 3 under control conditions; 48 ± 2 after t-ACPD + AMPA. *N.D.*, not detectable.

cells in the presence of glutamate receptor antagonists (Fig. 1*D*). Fig. 1*E* shows the $[Ca^{2+}]_i$ responses induced by the superfusates collected under static bath conditions (*a*) or during mechanical stimulation (*b*). The $[Ca^{2+}]_i$ responses were completely prevented when the conditioned medium was treated with apyrase for 10–15 min (Fig. 1*F*), thus indicating that ATP was the involved bioactive compound. Analysis of lactate dehydrogenase release revealed no significant difference between the extracellular media collected under static bath conditions or during mechanical stimulation (lactate dehydrogenase activity: control, 4.87 ± 0.69 units/liter; mechanically stimulated, 6.27 ± 1.2 units/liter, $n = 4$, $p = 0.37$; Triton X-100-treated, 247 ± 11.3, $n = 4$, $p < 0.001$), thus excluding ATP leakage caused by shear damage. The lack of cell damage was also confirmed by the exclusion of Trypan Blue from the mechanically stimulated astrocytes (data not shown).

Regulated ATP Release from Astrocytes—We used the two bioassays to obtain insights into the mechanisms that control ATP release from primary astrocytes on different kinds of stimulation. To investigate the possible calcium dependence of ATP release, astrocytes were mechanically stimulated after 45 min treatment with the intracellular calcium chelator BAPTA/AM (10 μM). Purine release was largely calcium-dependent, as the response in ATP reporter cells was significantly attenuated when the medium was collected from BAPTA-treated astrocytes (off-site bioassay, Fig. 1*E*, *c*). BAPTA treat-

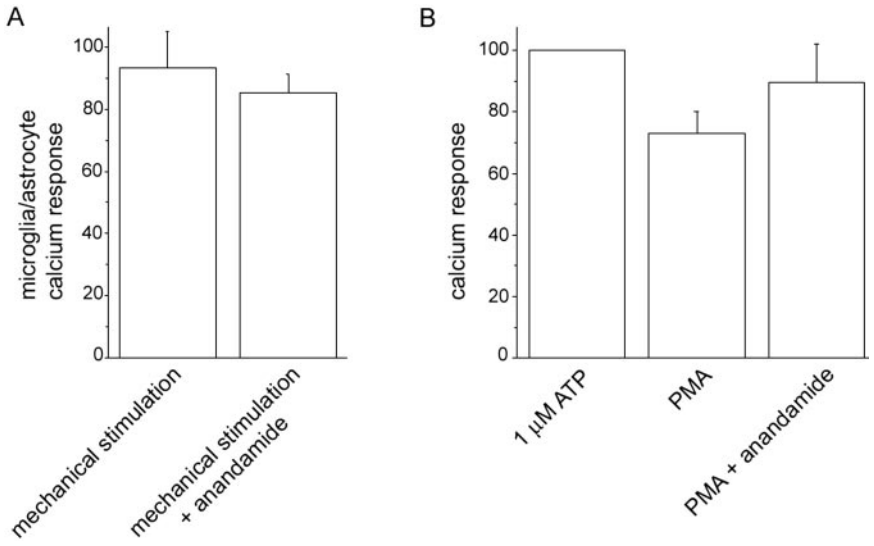

FIG. 2. **The evoked release of ATP from astrocytes is gap-junction-independent.** *A*, quantitative analysis of mechanically evoked ATP release from astrocytes in the presence or absence of anandamide, based on the on-line ATP bioassay. Histograms show mean values ± S.E. of $[Ca^{2+}]_i$ responses in microglia normalized to $[Ca^{2+}]_i$ increases in mechanically stimulated astrocytes, with or without the gap-junction blocker anandamide (control = 93.3 ± 9.3; n = 14; anandamide-treated = 82.35 ± 6.3; n = 14; $p > 0.1$. *B*, quantitative analysis of ATP release upon PMA stimulation with and without anandamide, based on the off-line ATP bioassay. Values represent percent changes (±S.E.) normalized to the $[Ca^{2+}]_i$ responses induced by 1 μM exogenous ATP (control 73.1 ± 7; n = 9; anandamide-treated = 89.6 ±12 n = 9; $p > 0.1$.

ment significantly reduced the calcium response to below baseline levels (Fig. 1*F*). Furthermore, ATP release was significantly increased by treatment with the potent secretagogues PMA (100 nM) (Fig. 1*G*) or with the glutamate receptor agonists AMPA (100 μM) and t-ACPD (100 μM) (Fig. 1*H*), which have been previously shown to stimulate glutamate secretion when simultaneously applied to astrocytes (31). On the basis of a standard dose-response curve of $[Ca^{2+}]_i$ response amplitude to different concentrations of exogenous ATP, the actual ATP concentration in the collected medium was estimated to be 130–290 nM under static bath and 550–700 nM after mechanical stimulation or secretagogue treatment. Determination of the ATP in the extracellular medium using the sensitive luciferin-luciferase bioluminescence assay revealed a 4.5 ± 0.4-fold (n = 3) increase in ATP release after PMA stimulation.

We then evaluated whether connexin hemichannels mediate ATP efflux from primary astrocytes upon stimulation. To test this hypothesis directly, we measured stimulated ATP release in the presence of the gap-junction blockers anandamide, which effectively uncouples astrocytes (11) or flufenamic acid, which has been recently used as connexin hemichannel blocker (21, 32). As shown in figure 2*A*, no significant reduction in $[Ca^{2+}]_i$ response was observed when the astrocytes were mechanically stimulated after 10–30 min incubation with 100 μM anandamide (on-line bioassay). Accordingly, $[Ca^{2+}]_i$ responses in ATP reporter cells to the medium conditioned by a PMA-stimulated astrocyte monolayer were unaffected by the presence of the gap-junction blocker (off-site bioassay) (Fig. 2*B*). To ensure that the doses of anandamide used in this study were appropriate to block gap-junction communication, FURA-2-loaded astrocytes were mechanically stimulated in the presence of the purinergic antagonist PPADS and the ATP-degrading enzyme apyrase with or without anandamide. When the extracellular pathway was inhibited by the purinergic blockers, anandamide completely prevented calcium signal propagation, thus indicating an efficient block of the gap-junction-mediated communication (wave propagation radius: PPADS, apyrase, without anandamide: 145.9 ± 11 μm, n = 4; PPADS, apyrase, with anandamide: 12.1 ± 6.4 μm, n = 5, $p < 0.01$). Furthermore, as blocking of gap-junctions may not be indicative of inhibition of connexin hemichannels, we tested whether anandamide blocks NAD^+ influx down a concentration gradient, which is known to occur through connexin hemichannels (33). An 86 ± 13% reduction of NAD^+ influx was caused by a 15-min preincubation with 100 μM anandamide. These data indicate that anandamide, which is effective in blocking nucleotide fluxes through connexin hemichannels, does not significantly impair ATP release evoked by astrocyte stimulation. Similarly, no significant reduction in ATP release, monitored as $[Ca^{2+}]_i$ response in microglial cells (on-line bioassay), was observed when the astrocytes were mechanically stimulated after 10–30 min incubation with 50 μM flufenamic acid (data not shown).

It has been reported in the literature that exposure of astrocytes to calcium-free medium facilitates ATP release and that this treatment promotes opening of connexin hemichannels (15, 21). Treatment of hippocampal astrocytes with calcium-free medium significantly increased basal ATP release, as detected by using the sensitive luciferin-luciferase assay (196 ± 15% increase upon controls). ATP release was significantly reduced (30 ± 1.8% reduction) in cultures incubated with the gap-junction inhibitor anandamide.

Subcellular Distribution of ATP in Cultured Astrocytes—Our data indicated the existence of a calcium-dependent, gap-junction blocker-insensitive release of ATP in primary cultures of hippocampal astrocytes, suggesting a vesicular purine storage. In line with this hypothesis, labeling of astrocytes with quinacrine fluorescence dye, which is known to stain high levels of ATP bound to peptides in large granular vesicles (24, 34) revealed the existence of an ATP-containing population of vesicular organelles, prominently localized in the perinuclear region of the astrocytes (Fig. 3*A*). This vesicular staining was reminiscent of the localization of secretogranin II (SgII), a well established marker of dense-core vesicles (35) recently detected in astrocytes (Ref. 25; Fig. 3*B*; also see Fig. 3*D*). To test directly whether ATP is contained in secretory granules, the astrocytes were analyzed by means of subcellular fractionation on sucrose equilibrium gradients, and the fractions were probed for ATP using the luciferin-luciferase bioluminescence assay. This analysis revealed the presence of a purine peak that completely overlapped SgII immunoreactivity, as well as a major peak in the lightest fractions that possibly represents cytosolic ATP (Fig. 3*C*). These data indicated that at least a portion of ATP is packaged in the secretory granules of hippocampal astrocytes.

A number of the proteins of the synaptic vesicle exocytotic machinery, including synaptobrevin/VAMPII (36), have been identified in astrocytes (26–28), and there is evidence suggesting the existence of synaptobrevin/VAMPII-positive vesicles in glial cells (37). The bulk of synaptobrevin/VAMPII was recovered in the light sucrose gradient subcellular fractions (fractions 9–13), and only a light trail of the synaptic vesicle marker overlapped the SgII- and ATP-containing fractions (fractions 14–18). A similar subcellular distribution of synaptobrevin/

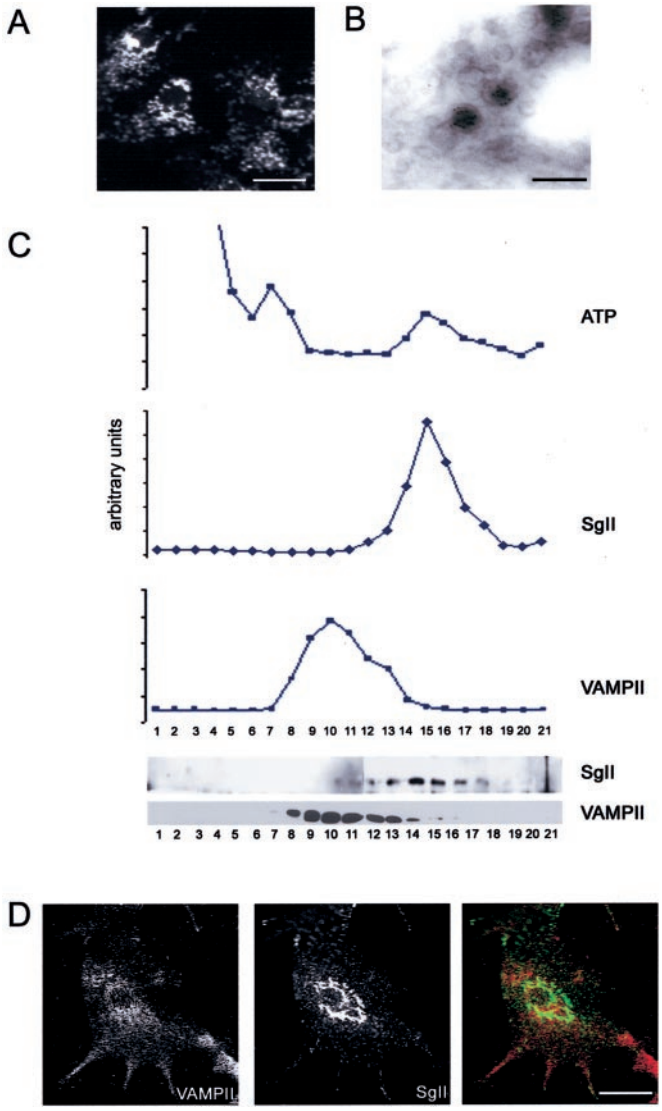

FIG. 3. **Subcellular distribution of ATP in astrocytes.** *A*, quinacrine staining in living astrocytes. Note the presence of dot-like structures dispersed in the cytoplasm, which suggest dye accumulation in vesicular organelles. *Bar*, 40.65 μm. *B*, electron micrograph showing dense-core vesicles immunolabeled for SgII (10-nm gold particles). Note the presence of unlabeled smaller vesicles in the ultrathin section. *Bar*, 0.16 μm. *C*, plot of ATP content and profile of SgII and synaptobrevin/VAMPII distribution in the sucrose gradient fractions. Note the presence of an ATP peak completely overlapping the SgII-immunoreactive fractions. An example of the distribution of SgII and synaptobrevin/VAMPII in the gradient fractions is shown *below* the graphs. *D*, double-immunofluorescence staining for SgII (*green*) and synaptobrevin/VAMPII (*red*). The merged image is shown in the *right panel*. *Bar*, 54.2 μm.

VAMPII *versus* SgII has been previously reported in neuroendocrine cells, which contain small synaptic-like vesicles and secretory granules (38–42).

The coexistence of two distinct types of vesicles in astrocytes is further supported by the immunofluorescence staining of cultures for synaptobrevin/VAMPII and SgII. As shown in Fig. 3*D*, synaptobrevin/VAMPII immunoreactivity (*red*) did not colocalize with the puncta of SgII staining (*green*) representing individual secretory granules, but appeared to reflect the distribution of a smaller vesicle population.

Bafilomycin A_1 Strongly Impairs ATP Storage and Reduces ATP Release—Studies on chromaffin cells indicate that ATP uptake in secretory granules requires an electrochemical proton gradient (43, 44), maintained by a v-ATPase that is selectively inhibited by bafilomycin A_1 (45, 46). To get insights into the mechanisms of ATP storage, astrocytes were treated with bafilomycin A_1 (1–4 μM) for 60 min and then probed for quinacrine staining. Virtually no fluorescent staining was detected within treated cells, as shown in Fig. 4 (*A–D*). A fluorescent granular pattern was recovered 30–60 min after washing (data not shown), thus suggesting a reversible action of the drug (47).

Consistent with the possibility that bafilomycin A_1 impairs ATP storage in secretory organelles, treatment with the v-ATPase inhibitor significantly impaired the calcium-evoked release of the purine, as indicated by the reduced $[Ca^{2+}]_i$ responses in adjacent microglia cells (Fig. 4*E*) (on-line bioassay) (66.3 ± 4.05% inhibition of microglial/astrocyte calcium response after bafilomycin A_1, $n = 19$, $p < 0.01$). No significant changes in mean astrocyte calcium responses were recorded in the presence of bafilomycin A_1 (see also Ref. 48), thus ruling out a possible interference of bafilomycin A_1 with the Ca^{2+} signal that is necessary to trigger ATP release (percent changes in Δ340/380 fluorescence ratio: 112.8 ± 12.7, $n = 20$, $p = 0.55$ data normalized to controls). Moreover, microglial $[Ca^{2+}]_i$ responses to exogenous ATP were not affected by bafilomycin A_1 treatment.

ATP Release Is Partially Sensitive to Tetanus Toxin—The possible existence of two classes of secretory organelles in astrocytes raised the question as to whether ATP and glutamate (the other main bioactive compound released by astrocytes) are stored in the same or different organelles. To clarify this point, the sensitivity of ATP release to TeNT was evaluated and compared with that of glutamate in the same intoxicated cells. If glutamate and ATP are stored in the same organelles, then the secretion of both messengers should be equally sensitive to the toxin. TeNT (100 nM, 24 h incubation) produced a massive, but not complete, cleavage of its molecular target synaptobrevin/VAMPII (Fig. 5, *A* and *B*).

To test the TeNT sensitivity of glutamate or ATP release, aliquots of medium conditioned by the same astrocyte monolayer before or after TeNT treatment were tested in parallel on neurons for glutamate detection (29, 30) or on astrocytes for ATP detection (Fig. 5*C*). In line with previous results (27, 31, 37, 48), TeNT treatment of the astrocyte monolayers considerably impaired both t-ACPD- and PMA-evoked glutamate release (Fig. 5*D*). On the contrary, only a partial reduction of ATP release was observed in the same conditioned medium after TeNT intoxication (Fig. 5*F*). A similar inhibition of ATP release from stimulated astrocytes was estimated by means of the on-line biological assay in astrocyte-microglia co-cultures after TeNT intoxication (32.8 ± 3.8% inhibition of microglial/astrocyte calcium response after TeNT treatment, $n = 15$, $p < 0.05$). No further inhibition of ATP release was produced by flufenamic acid on TeNT-intoxicated astrocytes (32 ± 1.8% inhibition in intoxicated astrocytes treated with flufenamic acid, $n = 34$).

TeNT treatment did not lead to a general reduction in the physiological integrity of astrocytes, which did not show any change in the baseline levels of calcium or sodium, thus indicating the preservation of ionic homeostasis (percent changes in the 340/380 fluorescence ratio after TeNT: calcium dye (FURA-2), 103.6 ± 2.95%, $n = 8$, $p < 0.05$; sodium dye (SBFI), 102.5 ± 1.98%, $n = 6$, $p < 0.05$, values normalized to controls). Furthermore, responsiveness to exogenous AMPA was not significantly altered (percent change in response after TeNT: 127 ± 8.1%, $n = 6$, $p = 0.68$, data normalized to controls), thus indicating that receptor trafficking was not significantly affected. The different sensitivity of glutamate and ATP release to the action of TeNT rules against the accumulation of the two messengers in the same intracellular exocytotic vesicles. Furthermore, the partial sensitivity of ATP release to TeNT fits the

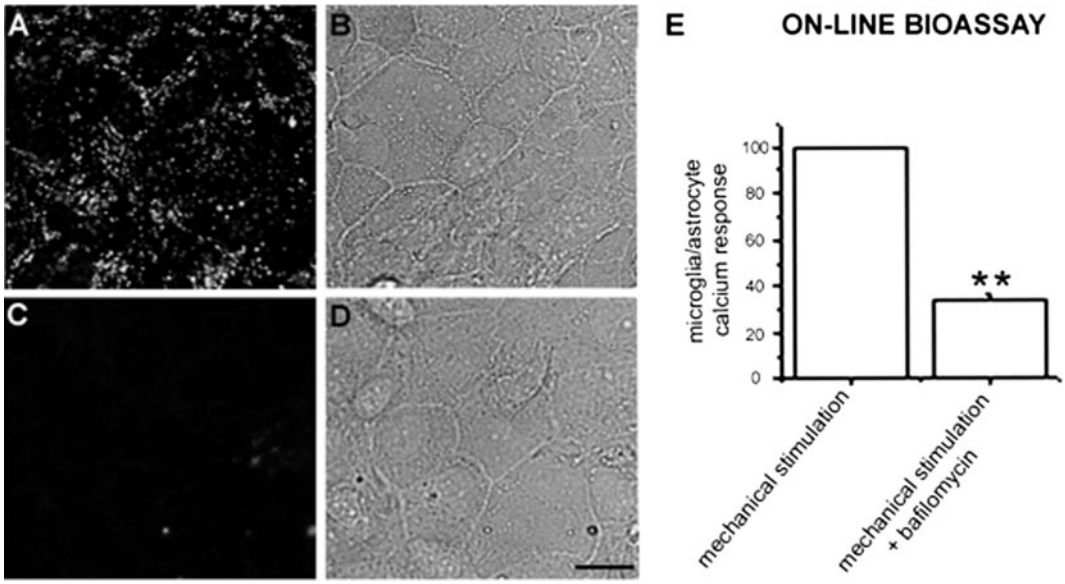

FIG. 4. **Effect of bafilomycin on ATP storage and release.** *A*, stores of ATP distributed in vesicular organelles visualized by quinacrine staining in living astrocytes. *C*, after treatment with bafilomycin A_1 virtually fluorescent granular staining was detected. *B* and *D*, differential interference contrast images of the same fields as *A* and *C*, respectively. *E*, quantitative analysis of mechanically evoked ATP release from astrocytes in the presence or absence of bafilomycin A_1, based on the on-line ATP bioassay. Histograms show mean values ± S.E. of $[Ca^{2+}]_i$ responses in microglia normalized to $[Ca^{2+}]_i$ increases in mechanically stimulated astrocytes in bafilomycin A_1-treated cultures (33.7 ± 2.69; $n = 18$; $p < 0.01$). *Bar*, 42.3 μm.

main storage of purine in secretory granules, which are known to be only partially sensitive to clostridial neurotoxins (42).

Release of Glutamate and ATP Can Be Differently Evoked—Phorbol esters are potent exocytotic stimuli that enhance regulated secretion from a variety of cell types including astrocytes. In line with its ability to stimulate the exocytosis of both synaptic vesicles (49) and secretory granules (25, 50), PMA significantly increased astrocyte ATP and glutamate release. Stimulations with PMA or t-ACPD, without any other glutamate agonists, were similarly effective in inducing the astrocyte release of glutamate (Fig. 6*A*), whereas t-ACPD was much less efficient than PMA in triggering ATP secretion (Fig. 6*B*). These data were confirmed by the direct evaluation of ATP and glutamate concentrations in the superfusates (glutamate, by HPLC: 33.5 ± 5 pM (control), 450 ± 6.8 pM (after t-ACPD treatment), 559 ± 7 pM (after PMA treatment); ATP, by luciferin-luciferase assay: 6–8-fold increase (after PMA stimulation), no increase (after t-ACPD stimulation)). It is worth noting that t-ACPD did not significantly increase SgII secretion (data not shown).

DISCUSSION

Although it is now widely established that ATP is an essential component of long-range calcium signaling in the nervous system (23) involved in the propagation of calcium waves (10, 14, 15, 18), the mechanisms regulating its release from glial cells have not been clarified yet. It has been suggested that ATP release from astrocytes may not be vesicular (13, 15, 48, 51) on the basis of three main lines of evidence: ATP release is tightly linked to connexin expression in C6 glioma cell lines; α-latrotoxin, a potent exocytotic stimulus, does not evoke ATP release (15); and calcium waves propagate among astrocytes in the presence of botulinum toxin (48), a blocker of vesicle fusion with the plasma membrane. In this study, we report an evoked, calcium-dependent ATP release from the secretory granules of primary astrocytes.

To investigate ATP storage and release mechanisms, we set up two bioassays for ATP detection in the culture system. The first bioassay was based on off-site measurements of ATP in superfusates collected from differently stimulated astrocytes and represents a modification of the method previously used by Guthrie *et al.* (10); the second is a novel on-line biological assay based on the ability of microglia co-cultured with astrocytes to respond to the ATP released from adjacent astrocytes with $[Ca^{2+}]_i$ transients (19). The absence of gap junctions between astrocytes and microglia (Ref. 52 and this study) and the absence of calcium-permeable glutamate receptors on microglial cells,[3] allow the evaluation of the $[Ca^{2+}]_i$ transients mediated by ATP, without considering either the glutamate- or gap-junction-mediated components. Even more important, this method also allows ATP detection at the astrocyte-releasing sites, thus avoiding dilution and degradation. Although the incomplete abrogation of the microglial calcium response by PPADS and apyrase could not exclude the minor contribution of other mechanisms, such as the nitric oxide-G-kinase signaling pathway (53), this assay is particularly well suited to investigate the mechanisms involved in purine release from glial cells.

The results of both assays indicated secretagogue- and calcium-dependent ATP release from astrocytes. The possible ATP efflux through connexin hemichannels does not account for the release triggered by PMA or mechanical stimulation. On the other hand, as previously reported (15, 21), we confirm that connexin hemichannels contribute to basal ATP release facilitated by exposure of the cultures to calcium-free medium. These data suggest the coexistence, in primary astrocytes, of mechanisms that mediate ATP secretion via connexin hemichannels or vesicular organelles depending on environmental conditions and on the activation of astrocytes.

To further test the possibility of vesicular storage, we used cell fractionation analysis. Luciferin-luciferase assays of the ATP content in subcellular fractions obtained using a sucrose equilibrium gradient showed that a significant amount is concentrated in the secretory granule-containing fractions. As previously demonstrated for chromaffin and neurosecretory granules (43, 54), ATP is accumulated in these organelles down an electrochemical proton gradient. The existence of dense core

[3] C. Verderio and M. Matteoli, unpublished data.

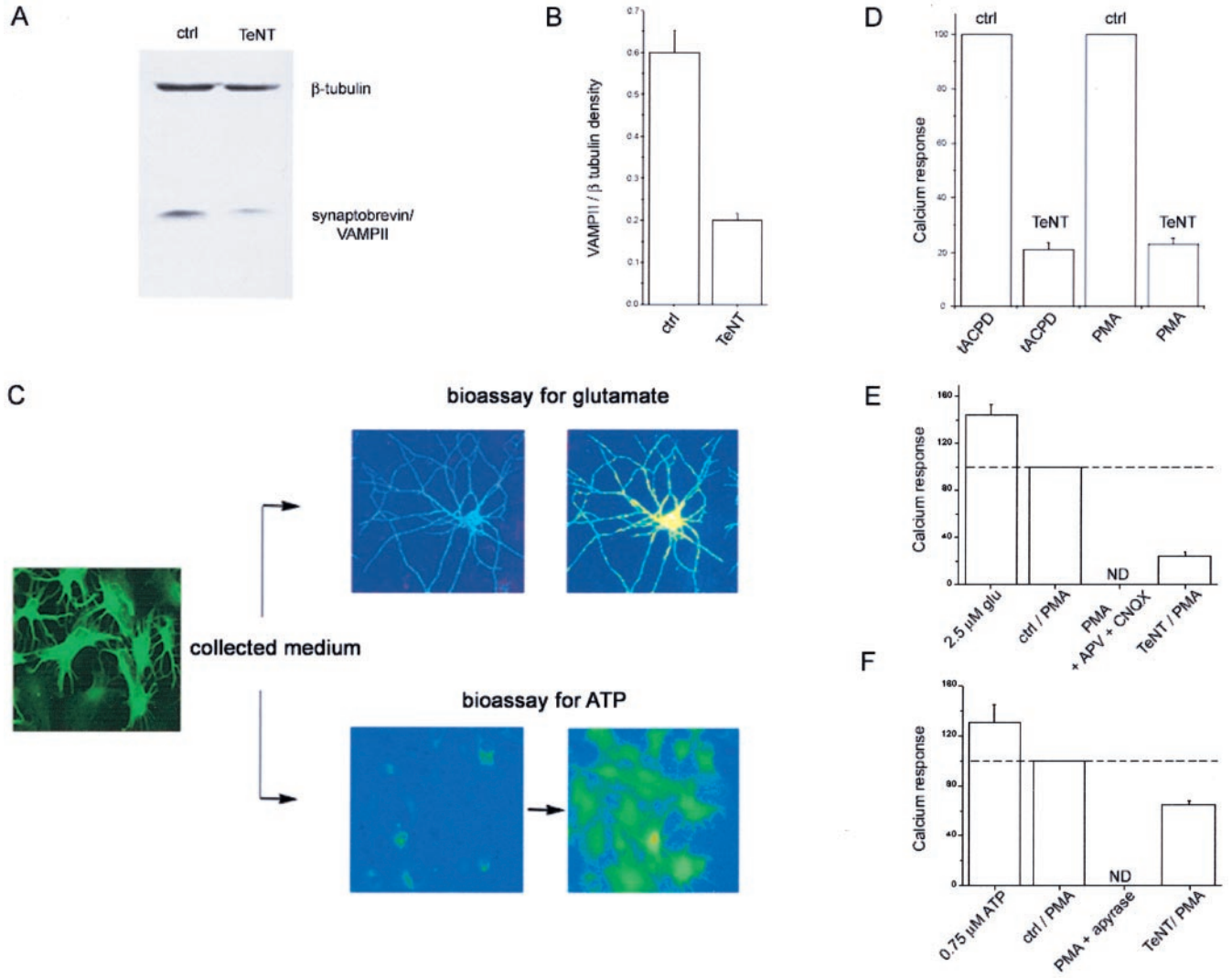

FIG. 5. **TeNT differently affects ATP and glutamate release from astrocytes.** *A*, Western blot analysis of control and TeNT-intoxicated cultured rat astrocytes shows that synaptobrevin/VAMPII is massively cleaved by the clostridial toxin. β–tubulin staining is shown as a standard. *B*, quantitative analysis of synaptobrevin/VAMPII content in control and intoxicated cultures. Synaptobrevin/VAMPII immunoreactivity is normalized to β-tubulin staining. *C*, schematic representation of glutamate and ATP measurements in medium conditioned by the same astrocyte monolayer. Aliquots of conditioned medium collected before and after TeNT intoxication were split into two parts and tested in parallel on glutamate reporter neurons and ATP reporter cells. *D*, quantitative analysis of the glutamate released by t-ACPD- or PMA-treated astrocyte monolayers before and after 24-h TeNT intoxication (t-ACPD: 21 ± 2.6% residual $[Ca^{2+}]_i$ response after TeNT, $n = 9$; PMA: 23 ± 2.2% residual $[Ca^{2+}]_i$ response after TeNT, $n = 11$). *E* and *F*, quantitative analysis of TeNT sensitivity of glutamate (*E*, 24 ± 3.5 residual $[Ca^{2+}]_i$ response after TeNT, $n = 24$) and ATP release (*F*, 65 ± 3% residual $[Ca^{2+}]_i$ response after TeNT, $n = 17$) from the same intoxicated astrocyte culture. The histograms in *D*, *E*, and *F* show percent changes (+S.E.) normalized to the $[Ca^{2+}]_i$ responses induced by medium conditioned by astrocyte monolayers before TeNT intoxication. *N.D.*, not detectable.

vesicles capable of undergoing regulated release has been previously reported in hippocampal astrocytes (25). The morphology and density of the vesicles are similar to those of the secretory granules found in neuroendocrine cells and, like them, contain the well known SgII marker of the regulated secretory pathway. Hippocampal astrocytes release SgII in response to secretagogues in a calcium-dependent manner. The PMA protein kinase C activator has been found to be particularly efficient in inducing regulated SgII release from astrocytes (25). More modest, but still significant, effects have been observed after treatment with bradykinin (25), whereas the t-ACPD metabotropic glutamate receptor activator had hardly any effect.[4] Similarly, ATP release was efficiently induced by PMA, but not by t-ACPD. Together with the results of the cell fractionation experiments, these findings clearly indicate that the dense core vesicles of hippocampal astrocytes co-store ATP with SgII. However, the existence of different purine pools could not be excluded because ATP was also detected in lighter fractions that are not immunoreactive to ER or mitochondrial markers (ribophorin and complex 3),[5] and whose identity has still to be defined.

Having found that astrocytic secretory granules contain ATP, the question arouse as to whether these organelles also store and release glutamate, the other main bioactive compound released by astrocytes. The ability of astrocytes to release glutamate via a calcium-dependent, vesicular mechanism has been clearly established (31, 37, 48). Our results suggest that glutamate is stored in vesicles other than the ATP- and SgII-containing large dense-core granules. The existence of two types of astrocytic secretory organelles was clearly shown by

[4] F. Calegari and P. Rosa, unpublished data.

[5] S. Coco, P. Rosa, M. Matteoli, and C. Verderio, unpublished data.

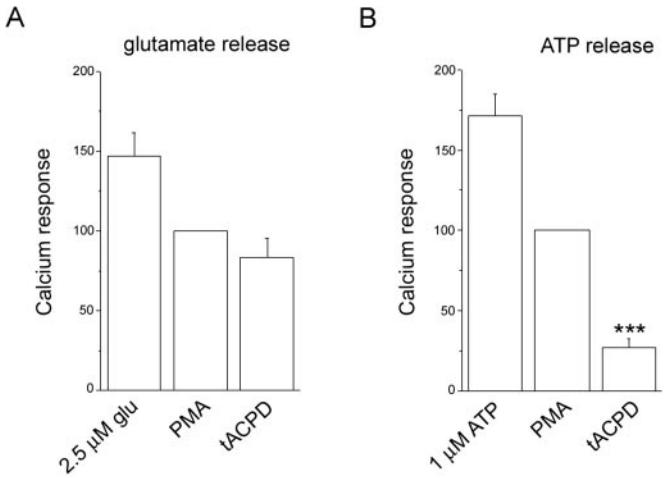

FIG. 6. **Glutamate and ATP release from astrocytes can be differently evoked by t-ACPD.** Quantitative analysis of glutamate (*A*) and ATP (*B*) levels in the extracellular medium conditioned by astrocytes upon PMA or t-ACPD stimulation. Histograms show percent changes (+S.E.) normalized to the $[Ca^{2+}]_i$ responses induced by the conditioned medium upon PMA treatment. (t-ACPD/PMA calcium response in glutamate reporter neurons: 83.4 ± 12 $p > 0.1$, $n = 8$; t-ACPD/PMA calcium response in ATP reporter astrocytes: 26 ± 5.5 $p < 0.01$, $n = 13$).

cell fractionation, immunocytochemistry and functional assays. Besides the clear lack of colocalization of SgII- and synaptobrevin/VAMPII-positive organelles revealed by immunofluorescence, the two markers were clearly differently distributed in the sucrose gradient fractions. The immunoreactivity profile for the two proteins was identical to that obtained in sucrose gradient fractions of PC12 cells (42) which are characterized by the presence of typical secretory granules storing SgII, and synaptic-like microvesicles containing the bulk of synaptobrevin/VAMPII. In line with the small pool of synaptobrevin/VAMPII in the fractions enriched in dense-core granules, it has been shown that calcium-evoked catecholamine secretion from PC12 cells is only about 35% inhibited by TeNT despite the nearly complete cleavage of synaptobrevin/VAMPII (42). This has been considered a typical feature of secretory granules and, in the past, led to the hypothesis of the existence of other v-SNARES (soluble NSF attachment protein receptors on the granule membrane) (42). We took advantage of the different sensitivity of the two types of vesicles to TeNT on the assumption that, if glutamate and ATP are co-stored in the same organelles, the secretion of both messengers should be equally reduced by TeNT. The evaluation of ATP and glutamate release from the same intoxicated cultures revealed that the TeNT-induced cleavage of synaptobrevin/VAMPII leads to the considerable impairment of glutamate exocytosis but only a partial reduction in ATP release. This finding is in line with previous reports indicating that TeNT treatment almost completely abolishes the astrocyte secretion of the low molecular weight messenger glutamate (31, 37, 55). It also supports the concept that glutamate is stored in a population of light vesicles in astrocytes which, like neuronal synaptic vesicles, contain the bulk of synaptobrevin/VAMPII. Furthermore, in addition to indicating that ATP and glutamate are stored in different organelles, the only partial sensitivity of ATP release to TeNT supports the hypothesis that ATP is mainly stored in secretory granules, as already indicated by subcellular fractionation analysis. The general inability of clostridial toxins to completely impair purine release explains a previous observation that calcium waves still propagate among astrocytes in the presence of botulinum toxin (48). Furthermore, the storage of ATP in large dense-core granules is also compatible with the finding that α-latrotoxin, a potent exocytotic stimulus purified from spider venom, does not evoke ATP release (15). It has been found that the stimulation of motor nerve terminals with α-latrotoxin potently induces the release of acetylcholine-containing vesicles, but is almost completely ineffective in causing the exocytosis of peptide-containing large dense-core vesicles (56).

The data reported here provide strong evidence for a vesicular storage and release of ATP. These findings, together with the previously reported connexin hemichannel-mediated ATP release, provide support for the coexistence of different pathways that may be differentially activated depending on the functional state of the astrocytes. The possibility that astrocytes may privilege one or the other pathway in different physiological or pathological conditions has intuitive appeal in explaining the plasticity of astrocytes in responding to the enviromental stimuli. Furthermore, the storage of ATP and glutamate in distinct vesicular organelles opens up the interesting possibility that their exocytosis is differently regulated. In line with this hypothesis, we found that the activation of metabotropic glutamate receptors efficiently induces glutamate exocytosis but not ATP release. It is well known that in neurons the exocytosis of synaptic and large dense-core vesicles is differentially regulated, with more potent stimuli being required to induce large dense-core vesicle fusion (57, 58). A more widespread analysis of the signals that may differentially affect the release of the two main astrocytic extracellular messengers is essential to improve our understanding of the mechanisms of communication among astrocytes and between them and other cell types in the nervous system.

Acknowledgments—We thank Prof. E. Fedele (University of Genova) for HPLC glutamate detection, Dr. S. Bruzzone and Prof. A. De Flora (University of Genova) for NAD^+ flux measurements, Prof. C. Montecucco (University of Padova) for the gift of tetanus neurotoxin, and U. Schenk for her help in some experiments. We thank Prof. F. Clementi (University of Milano) for helpful discussions and comments.

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