Cellular/Molecular

Mitochondria Modulate Ca^{2+}-Dependent Glutamate Release from Rat Cortical Astrocytes

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Vesicular glutamate release from astrocytes depends on mobilization of free Ca^{2+} from the endoplasmic reticulum (ER), and extracellular space to elevate cytosolic Ca^{2+} (Ca^{2+}_{cyt}). Although mitochondria in neurons, and other secretory cells, have been shown to sequester free Ca^{2+} and have been implicated in the modulation of Ca^{2+}-dependent transmitter release, the role of mitochondria in Ca^{2+}-dependent glutamate release from astrocytes is not known. A pharmacological approach was taken to manipulate Ca^{2+} accumulation in mitochondria and thereby affect Ca^{2+}_{cyt} of solitary astrocytes in response to mechanical stimuli. Ca^{2+}_{cyt} responses and levels of glutamate release were measured optically in parallel experiments using a fluorescent Ca^{2+} indicator and an enzyme-linked assay, respectively. It was observed that inhibiting mitochondrial Ca^{2+} accumulation is correlated to increased Ca^{2+}_{cyt} and glutamate release, whereas enhancing mitochondrial Ca^{2+} accumulation is correlated to decreased Ca^{2+}_{cyt} and glutamate release. These observations suggest that, in addition to the activity of ER and plasma membrane ion channels, mitochondria modulate Ca^{2+}_{cyt} dynamics in astrocytes and play a role in Ca^{2+}-dependent glutamate release from astrocytes.

Key words: **mitochondria; astrocyte; calcium; glutamate; release; exocytosis**

Introduction

Ca^{2+}-dependent glutamate release by astrocytes modulates synaptic communication between neurons (Araque et al., 1999). This has been initially demonstrated in cell cultures (Araque et al., 1998), and since then in various tissue preparations as well, including the retina (Newman, 2003b), hippocampus (Kang et al., 1998; Angulo et al., 2004; Fellin et al., 2004), and thalamus (Parri et al., 2001). In recent years, evidence also implicates astrocytic glutamate release in neural dysfunction such as epilepsy. Chemically induced epileptiform activity in neurons promotes cytosolic Ca^{2+} (Ca^{2+}_{cyt}) increase in neighboring astrocytes through activation of metabotropic glutamate receptors (Ding et al., 2007). As a consequence, heightened astrocytic Ca^{2+} excitability causes additional glutamate release that activates extrasynaptic *N*-methyl-D-aspartic acid receptors to drive neuronal activity (Kang et al., 2005; Tian et al., 2005).

In astrocytes, Ca^{2+} necessary for exocytotic glutamate release originates primarily from inositol 1,4,5-trisphosphate (IP_3)- and caffeine/ryanodine-sensitive stores of the endoplasmic reticulum (ER) (Hua et al., 2004). Mitochondria provide the primary Ca^{2+} buffer in cultured chromaffin cells (Herrington et al., 1996; Babcock et al., 1997), and dorsal root ganglion neurons (Thayer and Miller, 1990; Werth and Thayer, 1994). When positioned near IP_3-gated channels, mitochondria buffer histamine-induced Ca^{2+} released by the ER and may facilitate the oscillatory property of free Ca^{2+}_{cyt} in stimulated astrocytes (Jou et al., 1996) and HeLa cells (Ishii et al., 2006). In addition, studies in *Xenopus* oocytes (Jouaville et al., 1995) and astrocytes (Simpson et al., 1998; Boitier et al., 1999) demonstrate that mitochondria regulate Ca^{2+} wave dynamics within the cytosol.

Studies in neurons (Tang and Zucker, 1997) and adrenal chromaffin cells (Giovannucci et al., 1999) implicate the importance of mitochondria in Ca^{2+}-dependent exocytosis. Although it is widely recognized that exocytotic/vesicular release of glutamate from astrocytes is Ca^{2+}-dependent, it has not been demonstrated whether mitochondrial Ca^{2+} handling plays a significant role in this process. Because Ca^{2+}_{cyt} increase is necessary for vesicular glutamate release from astrocytes (Parpura and Haydon, 2000), we tested whether mitochondrial buffering affects Ca^{2+}_{cyt} and in turn regulates glutamate release from astrocytes. In the present study, we took a pharmacological approach to manipulate mitochondrial Ca^{2+} accumulation and thereby modulate the transient Ca^{2+} increases in solitary cortical astrocytes. In parallel, we studied the effect of pharmacological agents on astrocytic glutamate release. The present study shows that mitochondria buffer transient increases in cytosolic Ca^{2+}, and consequently modulate Ca^{2+}-dependent glutamate release from cultured cortical astrocytes. These findings suggest a possible mechanism by which astrocytes could regulate the tripartite synapse (Araque et al., 1999).

Materials and Methods

Astrocyte cultures. A modified culture method (Parpura et al., 1995; Hua et al., 2004) was used to grow solitary astrocytes on permissive substrate

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[1 mg/ml polyethyleneimine (PEI); Sigma-Aldrich]. Visual cortices from 1- to 2-d-old Sprague Dawley rats were dissected, and after enzymatic treatment and mechanical dissociation, cells were seeded into culture flasks containing culture medium composed of α-MEM (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), 20 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 14 mM sodium bicarbonate, penicillin (100 IU/ml), and streptomycin (100 μg/ml), pH 7.35. After 1 h, cells attached to the culture flasks were washed and then provided fresh media. The cells were maintained at 37°C in a 95% air/5% CO_2 incubator for 5–7 d to allow for growth and proliferation to ~60% confluency. At that juncture, the cell cultures were purified for astrocytes (McCarthy and de Vellis, 1980), which were then plated onto round glass coverslips (12 mm in diameter) coated with PEI and maintained in a 37°C incubator until used in experiments after 3–4 d. The purity of astrocytic culture (>99%) was confirmed twofold: (1) by indirect immunocytochemistry using anti-glial fibrillary acidic protein antibody and (2) by visualization of accumulation of a dipeptide, β-Ala-Lys, conjugated to 7-amino-4-methylcoumarin-3-acetic acid, as described previously (Montana et al., 2004).

Pharmacological agents. Concentration and preincubation times for each pharmacological agent were adapted from literature (White and Reynolds, 1995; Jou et al., 1996; Baron and Thayer, 1997; Zhong et al., 2001; Bambrick et al., 2006; Wu et al., 2007): 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157) (50 μM; 5 min; Tocris Bioscience), cyclosporin A (CsA) (20 μM; 5 min; Alexis Biochemical), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (1 μM; 3 min; Sigma-Aldrich), ruthenium 360 (Ru360) (10 μM; 30 min; Calbiochem), and oligomycin (8 ng/ml; 30 min; Sigma-Aldrich). These agents were applied onto astrocytes in external solution, pH 7.35, containing the following (in mM): 140 sodium chloride, 5 potassium chloride, 2 calcium chloride, 2 magnesium chloride, 10 HEPES, and 5 glucose. Before calcium ion imaging and assessment of glutamate release, the astrocytes were preincubated in the pharmacological agent at room temperature (22–25°C) at the prescribed time stated above, and were kept bathed in the agents during the entire subsequent experimental procedure lasting 200 s.

Cellular and mitochondrial staining. The effects of the pharmacological agents on cell viability and on mitochondrial morphology were monitored using the vital stain calcein (Ni et al., 2005) and 4-(4-(diethylamino)styryl)-methylpyridium (4-Di-2-ASP) (Parpura et al., 1993), respectively. Astrocytes were loaded with calcein acetoxymethyl (AM) ester (1 μg/ml; Invitrogen) with the addition of 0.025% w/v pluronic acid (Invitrogen) to aid dispersion in complete culture medium, at 37°C for 10 min. After deesterification of the calcein AM for 10 min in external solution at room temperature, the astrocytes were incubated in 4-Di-2-ASP (10 μM; Invitrogen) for 3 min at room temperature. After subsequent washes with external solution, the astrocytes were incubated in the pharmacological agents. Calcein and 4-Di-2-ASP fluorescence emissions were visualized with standard fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) filter sets (Chroma), respectively. It should be noted that some 4-Di-2-ASP fluorescence emission can be recorded using the FITC filter set, although this bleed-through is insufficient to affect our calcein measurements. Calcein fluorescence emission is expressed as a ratio of background subtracted fluorescence at testing time (F) over baseline fluorescence of cells at rest (F_o), later being an average of five initial images.

Ca^{2+} imaging. Cytosolic Ca^{2+} levels of cultured astrocytes were measured using the Ca^{2+} indicator fluo-3 as described previously (Parpura et al., 1994; Hua et al., 2004). Briefly, the astrocytes were loaded with fluo-3 AM (10 μg/ml; Invitrogen)/pluronic acid (0.025% w/v) in external solution for 30 min at room temperature. Afterward, the cells were incubated in fresh external solution for 30 min at room temperature to allow deesterification of the fluo-3 AM, and were further incubated with pharmacological agents. Coverslips were mounted onto a recording chamber, and astrocytes were visualized with a standard FITC filter set (Chroma). All imaging data were prepared by subtracting the region of interest, soma of dye-loaded astrocytes, by the background fluorescence of regions containing no cells. Fluorescence data were expressed as dF/F_o (percentage) with the cell baseline fluorescence (F_o) representing the average of the first five images before mechanical stimulation and dF representing the change in fluorescence emission. At least three independent cultures, with equal number of cells within different treatment groups, were used to compare the effects of the pharmacological agents with control.

Extracellular glutamate imaging. Ca^{2+}-dependent glutamate release from astrocytes was measured using the L-glutamate dehydrogenase (GDH) (Sigma-Aldrich)-linked assay as previously described (Innocenti et al., 2000; Hua et al., 2004; Montana et al., 2004). PEI-coated coverslips containing cultured astrocytes were mounted onto a recording chamber, and the astrocytes were incubated in external solution with pharmacological agents. A set of images containing the cell of interest were taken in a sham run to correct for reduction of fluorescence caused by photobleaching. In the experimental run, the external solution was exchanged with fresh external solution additionally containing 1 mM β-nicotinamide adenine dinucleotide (NAD^+) (Sigma-Aldrich), 55–61 IU/ml GDH, and the pharmacological agent. The assay was visualized with a standard DAPI (4′,6-diamidino-2-phenylindole) filter set (Nikon). When released, glutamate is oxidized to α-ketoglutarate by GDH, whereas bath supplied NAD^+ is reduced to NADH, a fluorescent product when excited by UV light. Fluorescence data were expressed as dF/F_o (percentage) with the baseline fluorescence (F_o) being the fluorescence of the media surrounding the astrocyte before mechanical stimulation. At least three independent cultures were used to compare the effect of the pharmacological agents with control. For each experimental set, equal numbers of cells from treatment groups were tested to minimize variability generated by different batches of GDH used throughout the study.

GDH activity assay. To account for the possibility that the pharmacological agents may have an effect on the activity of the GDH in the media surrounding the astrocytes, an assay was performed using a spectrophotometer (Genequant Pro) and NADH absorbance as a measure of GDH activity. The assay solution contained normal external solution supplemented with NAD^+ (1 mM), glutamate (100 μM), and ~59 IU/ml GDH. The concentrations of pharmacological agents were identical with those used for Ca^{2+} and glutamate imaging. NADH produced from the reaction was monitored by its absorbance at 320 nm. This assay showed that, at the 5 min interval, which allowed the reaction to reach a steady-state level, there was no significant difference in NADH absorbance in solutions containing NAD^+, glutamate, GDH, and the various agents: control, 0.39 ± 0.03 (mean ± SEM); CGP37157, 0.37 ± 0.03; CsA, 0.32 ± 0.02; FCCP, 0.38 ± 0.02; Ru360, 0.32 ± 0.02; and oligomycin, 0.42 ± 0.03 (one-way ANOVA, $p_{(5,50)} = 0.17$).

Mechanical stimulation. To elicit cytosolic Ca^{2+} increase in the astrocytes and subsequent release of glutamate, we used a patch pipette to deliver mechanical contact (Charles et al., 1991). This particular method allows spatiotemporal control of the stimulus application, without affecting plasma membrane integrity (Hua et al., 2004). The patch pipette was lowered onto the astrocytes with a micromanipulator (Narishige) and contact with membrane was monitored by measuring the change of pipette resistance using a patch-clamp amplifier (PC-ONE; Dagan) that delivered −20 mV, 10 ms square pulses at 50 Hz. Pipette resistances measured 1.9–4.8 MΩ before contact with the astrocytes, which increased to 2.1–6.6 MΩ during contact, lasting <1 s, with the astrocytes.

Secretory vesicle pH assay. The effect of pharmacological agents on luminal pH of secretory vesicles were assessed by the reporter protein, synapto-pHluorin (Sankaranarayanan et al., 2000). We transfected astrocytes growing on four PEI-coated coverslips inlaid in Petri dishes (35 mm in diameter) containing 1 ml of culture medium with a plasmid (0.25 μg/ml) encoding for superecliptic synapto-pHluorin (provided by Dr. James E. Rothman, Memorial Sloan-Kettering Cancer Center, New York, NY) using TransIT-293 transfection reagent (2 μl; Mirus) as previously described (Montana et al., 2004). We compared the fluorescence of transfected cells, visualized with a FITC filter set, when treated with the pharmacological agents in external solution, pH 7.35. As a positive control for the ability of synapto-pHluorin to detect intravesicular pH, we incubated transfected cells with bafilomycin A1 (5 μM; 30 min; Sigma-Aldrich) to alkalinize vesicles. Additionally, we placed transfected cells after each trial in high pH external solution, pH 8.5, which alkalinizes the cytosol and the lumen of vesicles.

Image acquisition and processing. An inverted microscope (TE 300; Nikon), equipped with differential interference contrast, wide-field fluorescence illumination and oil-immersion objectives, was used in all experiments. For glutamate imaging, we used a 40× SFluor objective [1.3 numerical aperture (NA); Nikon], whereas all other experiments were done using a 60× Plan Achromatic objective (1.4 NA; Nikon). Images were acquired using a CoolSNAP-HQ cooled charge-coupled device (CCD) camera (Roper Scientific) driven by V++ imaging software (Digital Optics Ltd.). All images shown in figures represent raw data with their pixel intensities within the dynamic range (0-4095) of the camera (CoolSNAP-HQ). For the glutamate release analysis, the dF/F_o of the treatment groups were ranked and normalized to allow comparisons between experimental batches and accommodate for variations in GDH concentration and culture conditions. Similar ranking of intracellular Ca^{2+} dynamics dF/F_o values was done for consistency.

Statistical analysis. Effects of the pharmacological agents on intracellular Ca^{2+} dynamics, on exocytotic glutamate release, and on the pH of the vesicular lumen were tested using one-way ANOVA, followed by Fisher's least significant difference (LSD) test. Similar analysis was used to assess cell viability, with the exception of the FCCP effect that was tested using Student's *t* test. Increase in fluo-3 and NADH fluorescence caused by mechanical stimulation was tested using paired *t* tests. Changes in synapto-pHluorin fluorescence because of exposure of astrocytes from normal to high pH external solution was tested by one-way ANOVA, followed by Tukey–Kramer's procedure.

Results

Effects of pharmacological agents on cell viability and mitochondrial networks in solitary cortical astrocytes

Although most of the pharmacological agents used in the present study have been used to study the role of mitochondria in $Ca^{2+}{}_{cyt}$ dynamics (White and Reynolds, 1995; Budd and Nicholls, 1996; Jou et al., 1996; Babcock et al., 1997; Ishii et al., 2006; Wu et al., 2007), we initially tested the effects of all the agents on astrocytic viability focusing on the integrity of the plasma membrane and mitochondrial network. It was important to assess the effect of the agent on the plasma membrane because compromised plasma membranes may alter the Ca^{2+} response, and subsequent vesicular glutamate release from astrocytes. We loaded solitary cortical astrocytes with calcein AM, a dye taken up only by living cells (Bischof et al., 1995; Crowe et al., 1995), and measured its retention as astrocytes were exposed to pharmacological agents. We acquired images of calcein-loaded solitary astrocytes before and after treatment with the agents. Simultaneously, we assessed the effects of the agents on mitochondrial morphology. Mitochondria are structurally heterogeneous, and form dynamic networks within mammalian cells that are subject to fusion and fission (Collins et al., 2002; Westermann, 2002). Depolarization of the inner mitochondrial membrane with protonophores, such as FCCP, inhibits fusion and promotes fragmentation of mitochondria in HeLa cells (Legros et al., 2002). To visualize the mitochondrial networks, we loaded astrocytes with the mitochondria-specific stain, 4-Di-2-ASP (Parpura et al., 1993), subsequent to loading with calcein AM. We acquired images of the 4-Di-2-ASP-stained mitochondria in the same astrocytes that were preloaded with calcein, before and after treatment with the pharmacological agents.

Calcein displayed good retention within astrocytes at rest, consistent with our previous observation (Malarkey et al., 2008). For example, at 5 and 30 min interval the retention was 88 ± 11 and 68 ± 11%, respectively ($n = 6$) (Fig. 1*A*,*G*). Astrocytes treated with FCCP (1 μM; 3 min; $n = 5$) exhibited similar retention as their time-matched controls (Fig. 1*B*,*G*) (Student's *t* test, $p = 0.69$). Similarly, astrocytes treated with CGP37157 (50 μM; $n = 6$) or with CsA (20 μM; $n = 6$) for 5 min displayed calcein fluorescence retention that was not significantly different from retention levels recorded in time-matched, untreated (control) astrocytes ($n = 5$) (Fig. 1*C*,*D*,*G*) (one-way ANOVA, $p_{(2,14)} = 0.37$). Furthermore, astrocytes treated with Ru360 (10 μM; $n = 6$), or oligomycin (8 ng/ml; $n = 6$) exhibited calcein fluorescence that was not different from measurements acquired from untreated time-matched astrocytes ($n = 6$) at 30 min (Fig. 1*A*,*E–G*) (one-way ANOVA, $p_{(2,15)} = 0.82$). However, applying ethanol (50% v/v) to the astrocytes, which disrupts membranes, caused a rapid loss of fluorescence with significant reduction in calcein fluorescence when compared with time-matched, untreated astrocytes at 1 min (data not shown) and 5 min (Fig. 1*G*) (one-way ANOVA, followed by Fisher's LSD; $p < 0.01$). Thus, pharmacological agents intended to use in testing the mitochondrial role in Ca^{2+}-dependent glutamate release do not affect plasma membrane integrity, a prerequisite for this study.

Simultaneous to calcein imaging, we assessed mitochondrial morphology in astrocytes coloaded with 4-Di-2-ASP. In untreated astrocytes, there was no drastic change in mitochondrial morphology over time as shown in the images of an untreated cell at 0 min and again at 30 min interval (Fig. 1*A*, top). However, some of the agents induced changes in mitochondrial morphology. FCCP fragmented mitochondria in all astrocytes tested ($n = 6$) (Fig. 1*B*). Similarly, in one-half of astrocytes (three of six tested) treated with oligomycin, we also observed fragmentation, along with a decrease in the fluorescence from mitochondria labeled by 4-Di-2-ASP (Fig. 1*F*). In contrast, CGP37157, CsA, and Ru360 treatments did not have any visible effect on the mitochondrial networks (Fig. 1*C–E*). Thus, at the fluorescence microscopy level, most of agents applied in this study did not grossly affect mitochondrial morphology. FCCP and oligomycin, however, fragmented astrocytic mitochondria, consistent with causing depolarization of the mitochondrial inner membrane, and blocking of ATP synthase, respectively (Legros et al., 2002).

Mitochondrial Ca^{2+} accumulation modulates mechanically induced increases in $Ca^{2+}{}_{cyt}$ in solitary astrocytes

Once we characterized the lack of action of pharmacological agents on the permeability of plasma membrane and confirmed the known action of a subset of the agents on mitochondrial morphology, we applied them to test for any effects on intracellular Ca^{2+} dynamics in astrocytes. These cells display transient increases in $Ca^{2+}{}_{cyt}$ arising from the release of free Ca^{2+} from the ER, and Ca^{2+} entry from the extracellular space (Hua et al., 2004; Malarkey et al., 2008). Previous studies in various cell types including astrocytes have shown that mitochondria participate in buffering these $Ca^{2+}{}_{cyt}$ spikes (Boitier et al., 1999; Hajnóczky et al., 1999; Collins et al., 2001). For example, depolarization of the inner mitochondrial membrane inhibits Ca^{2+} accumulation in mitochondria (Jou et al., 1996; Boitier et al., 1999). In the present study, we assessed the ability of mitochondria in astrocytes to modulate $Ca^{2+}{}_{cyt}$ by manipulating both mitochondrial Ca^{2+} influx and efflux mechanisms using a pharmacological approach. To increase or prolong free Ca^{2+} accumulation by mitochondria, we used CGP37157 (50 μM; 5 min) and CsA (20 μM; 5 min). CGP37157 blocks Ca^{2+} efflux through the mitochondrial Na^+/Ca^{2+} exchanger (Collins et al., 2001; Zhong et al., 2001), whereas CsA increases mitochondrial Ca^{2+} accumulation in digitonin-permeabilized cells (Bambrick et al., 2006) via its inhibitory action on mitochondrial permeability transition pore formation. To decrease Ca^{2+} handling by mitochondria, we depolarized them by disrupting the H^+ gradient of the inner membrane with the protonophore FCCP (1 μM; 3 min) (White and Reynolds,

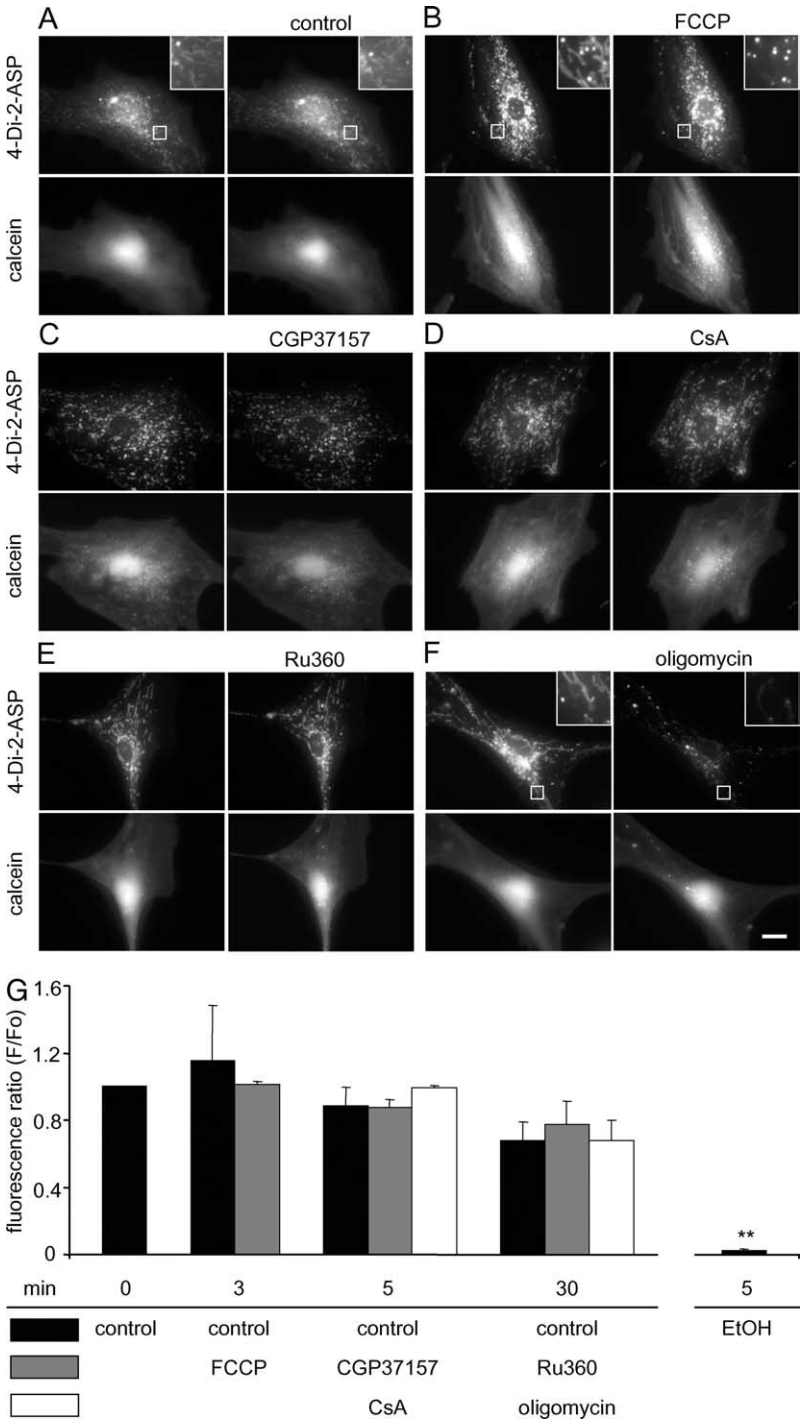

Figure 1. Mitochondrial network appearance and calcein retention in solitary cortical astrocytes treated with pharmacological agents. Astrocytes were incubated with the mitochondrial stain 4-Di-2-ASP and the vital stain calcein AM. The top two images in ***A–F*** show 4-Di-2-ASP-stained mitochondria as seen through the TRITC emission filter, whereas the bottom two images show the calcein fluorescence of the same cells as seen through the FITC emission filter. The left images of the panels depict the cells before treatment with the agent, whereas the right images of the panels depict the same cells after treatment. Cells were incubated in external solution without pharmacological agent (30 min; control) (***A***) or with various pharmacological agents: FCCP (1 μM; 3 min) (***B***), CGP37157 (50 μM; 5 min) (***C***), CsA (20 μM; 5 min) (***D***), Ru360 (10 μM; 30 min) (***E***), or oligomycin (8 ng/ml; 30 min) (***F***). Treatment of astrocytes with FCCP and oligomycin, unlike with the other agents, caused fragmentation of the mitochondrial network. ***G***, Bar graph of the average (+SEM) fluorescence ratio of calcein before (F_o) and after (F) incubation of astrocytes with pharmacological agents for various amounts of time (5–6 astrocytes in each group); time-matched control astrocytes were incubated in external solution lacking any agent. Statistical comparisons were made between sham run (control) and agent-treated astrocytes at the same time period (3, 5, or 30 min); agents had no significant effect on calcein retention. To the contrary, astrocytes treated with ethanol (EtOH) (50% v/v; 5 min) showed significant reduction in calcein retention (one-way ANOVA, followed by Fisher's LSD test, $^{**}p < 0.01$). The boxed areas (each 10×10 μm) in ***A***, ***B***, and ***F*** are displayed enlarged in top right corner insets. Scale bar, 20 μm.

1995; Collins et al., 2000; Ishii et al., 2006). We also used the Ca^{2+} uniporter blocker Ru360 (10 μM; 30 min) to prevent entry of free Ca^{2+} into the mitochondrial matrix (Matlib et al., 1998; Wu et al., 2007). Because FCCP and Ru360 can also deplete ATP, we used oligomycin (8 ng/ml; 30 min) as a control to inhibit ATP synthesis; oligomycin does not inhibit mitochondrial Ca^{2+} accumulation when substrate oxidation is functional (Lehninger et al., 1967).

We used solitary astrocytes and mechanical stimulation to minimize the effects of intercellular astrocytic communication that could alter Ca^{2+} dynamics (Hua et al., 2004; Montana et al., 2004). This stimulation causes an increase in cytosolic Ca^{2+} concentration in astrocytes by transfer of this ion from internal stores, although external Ca^{2+} plays a role (Hua et al., 2004), which can be mediated via store-operated channel activity (Malarkey et al., 2008). Astrocytes were loaded with the Ca^{2+} indicator fluo-3. To apply the mechanical stimulus, we established contact between a solitary astrocyte and a patch pipette under control of a patch-clamp amplifier. This direct stimulus caused an increase in intracellular Ca^{2+} levels (Fig. 2*A*) ($n = 75$; peak $dF/F_o = 390 \pm 13\%$; paired t test, $p < 0.01$), the peak of which corresponded to a Ca^{2+} accumulation of $\sim$2.2 μM over baseline ($\sim$75 nM), as determined using calibration of fluo-3 in parallel experiments as described previously (Malarkey et al., 2008). Astrocytes treated with CsA ($n = 15$) or CGP37157 ($n = 15$) exhibited lower Ca^{2+}_{cyt} responses than control astrocytes, as evidenced by attenuation of the peak response and decrease in the cumulative response (one-way ANOVA followed by Fisher's LSD test, $p < 0.01$ for peak and $p < 0.05$ for cumulative responses). In contrast, astrocytes treated with FCCP ($n = 15$) or Ru360 ($n = 15$) exhibited significantly enhanced peak and cumulative responses (one-way ANOVA followed by Fisher's LSD test, $p < 0.01$) when compared with untreated astrocytes (Fig. 2). Astrocytes treated with oligomycin ($n = 15$) showed a marginal increase in peak response (one-way ANOVA followed by Fisher's LSD test, $p < 0.05$), but their cumulative responses did not differ from those obtained from control astrocytes (Fig. 2).

Our data demonstrating that FCCP and Ru360 increase Ca^{2+}_{cyt} are consistent with previous studies (Matlib et al., 1998; Ishii et al., 2006). It appears that this increase in Ca^{2+}_{cyt} is a product of inhibition

of Ca^{2+} accumulation in mitochondria. This strongly suggests that, in cortical astrocytes, mitochondria are the immediate buffer to increases in Ca^{2+}_{cyt}. In conjunction, our data showing that CGP37157 and CsA decrease Ca^{2+}_{cyt} add additional support to such mitochondrial role. Decreasing Ca^{2+} extrusion from mitochondria or increasing mitochondrial Ca^{2+} accumulation lowered Ca^{2+}_{cyt}. The effects of FCCP and Ru360 on Ca^{2+}_{cyt} peak response, although unlikely, could in part be mediated by depletion of ATP because similar action was mediated by oligomycin; their enhancing effects on cumulative responses, however, diverge from oligomycin and hence are unlikely attributable to depletion of ATP.

Mitochondrial Ca^{2+} handling modulates glutamate release from solitary astrocytes

Having determined that cortical astrocytes in culture use mitochondria in modulation of mechanically induced Ca^{2+}_{cyt} responses, we asked whether mitochondrial Ca^{2+} handling plays a role in Ca^{2+}-dependent exocytotic release of glutamate from astrocytes. Ca^{2+} released from IP_3- and caffeine/ryanodine-sensitive ER stores into cytosol mediates mechanically induced exocytotic glutamate release from astrocytes (Hua et al., 2004); depleting the ER of Ca^{2+}, or blocking release of Ca^{2+} from IP_3- and caffeine/ryanodine-sensitive stores decreased glutamate release. To address whether the Ca^{2+}_{cyt} changes induced through manipulation of mitochondrial Ca^{2+} accumulation affect glutamate release, we measured glutamate release from astrocytes with an enzyme-linked assay. NADH fluorescence of the solution around mechanically stimulated astrocytes was measured in the presence of NAD^+ and GDH (Nicholls et al., 1987; Ayoub and Dorst, 1998). When glutamate is released from astrocytes into the extracellular space and immediately converted to α-ketoglutarate by GDH, NAD^+ is concomitantly reduced to NADH. Stimulating astrocytes by mechanical contact caused glutamate release as indicated by an increase in NADH fluorescence, reporting on extracellular glutamate levels, around the astrocytes (Fig. 3*A*) ($n = 44$; peak $dF/F_o = 49 \pm 5\%$; paired t test; $p < 0.01$); the peak fluorescence increase corresponds to extracellular glutamate accumulation of $\sim$1.5 μM when using the calibration of GDH-linked assay, as described previously (Innocenti et al., 2000). The pharmacological agents that tamper with mitochondria mainly affected glutamate release from astrocytes (Fig. 3), as one would predict from their actions on Ca^{2+} dynamics. Hence, we find that CGP37157-treated astrocytes ($n = 11$) had significantly reduced the peak and cumulative values of released glutamate when compared with untreated, control astrocytes (Fig. 3*B*,*C*) (one-way ANOVA followed by Fisher's LSD test, $p < 0.01$). Ru360-treated astrocytes ($n = 11$) exhibited significant increases in the peak and cumulative values for glutamate release (Fig. 3*B*,*C*) (one-way ANOVA followed by Fisher's LSD test, $p < 0.01$). However, there were some effects that we would not predict from the effects that agents exerted on Ca^{2+} dynamics. For example, although CsA-treated astrocytes ($n = 11$) show a trend in the reduction of peak values of glutamate release, it was insignificant, unlike the effect of CsA on the peak Ca^{2+}_{cyt} changes that was significant. It should be noted, however, that CsA effects on the cumulative values for Ca^{2+}_{cyt} and glutamate release were in good agreement, because both measurements showed significant reductions. This relatively minor discrepancy in predicted glutamate release based on the effect of the agent on Ca^{2+}_{cyt} changes, could perhaps be attributable to an inherent limitation of our approach using parallel measurements of Ca^{2+}_{cyt} dynamics and glutamate release. However, such explanation could not hold for the effects of FCCP and oligomycin on glutamate release. FCCP caused significant reductions in both the peak and cumulative values of glutamate release from astrocytes ($n = 11$) (Fig. 3*B*,*C*) (one-way ANOVA, followed by Fisher's LSD test, $p < 0.01$), although this agent enhanced Ca^{2+}_{cyt} responses (compare Figs. 2, 3). Similar variations were observed with oligomycin, which significantly reduced the peak and cumulative glutamate release from astrocytes ($n = 11$) (Fig. 3*B*,*C*) (one-way ANOVA, followed by Fisher's LSD test, $p < 0.01$), whereas corresponding Ca^{2+}_{cyt} changes were signifi-

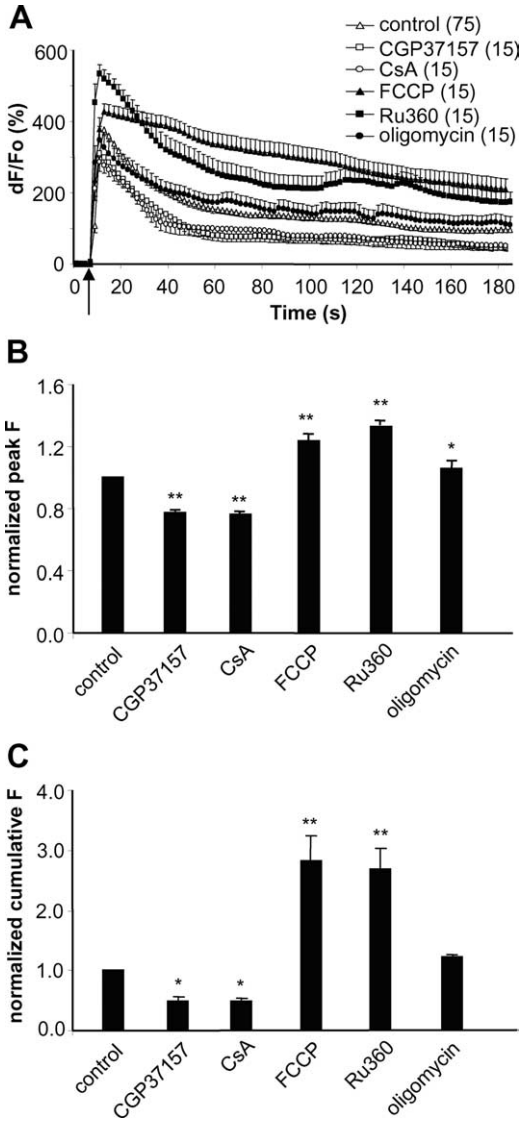

Figure 2. Pharmacological agents targeting mitochondria modulate mechanically induced Ca^{2+}_{cyt} increases in solitary astrocytes. ***A***, Average kinetics of fluo-3 fluorescence, reporting on changes in Ca^{2+}_{cyt}, in solitary astrocytes treated with pharmacological agents in response to mechanical stimulation. The points and bars represent means $\pm$ SEMs of measurements from individual astrocytes (numbers in parentheses) expressed as dF/F_o (percentage); SEMs are shown in single directions for clarity. The arrow represents the time when mechanical stimulation was applied to the cells. ***B***, Normalized fluo-3 peak fluorescence (F) values of mechanically stimulated astrocytes. When treated with CGP37157 or CsA, astrocytes had significantly lower peak fluorescence than control. Astrocytes treated with FCCP, Ru360, or oligomycin had significantly higher peak fluorescence than control. ***C***, Normalized fluo-3 cumulative fluorescence (F) values obtained from mechanically stimulated astrocytes. Pharmacological agents had similar effects as in ***B***, with the exception of oligomycin that did not alter the cumulative responses. The asterisks denote significant change compared with control group (one-way ANOVA, followed by Fisher's LSD test, $*p < 0.05$, $**p < 0.01$).

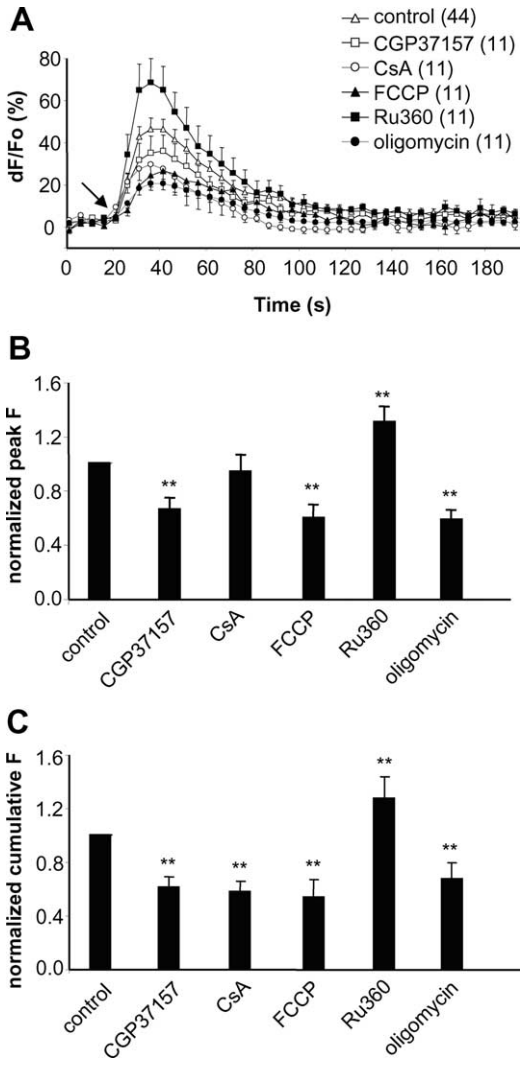

Figure 3. Pharmacological agents affecting Ca^{2+} accumulation in mitochondria modulate mechanically induced exocytotic glutamate release from solitary astrocytes. ***A***, Time lapse of NADH fluorescence, reporting on glutamate release. Mechanical stimulation of solitary astrocytes caused glutamate release (open triangles) that was affected by various pharmacological agents. The points and bars represent means ± SEMs of measurements from individual astrocytes (numbers in parentheses) expressed as dF/F_0 (percentage); SEMs are shown in single directions for clarity. The arrow represents the time when mechanical stimulation was applied to the cells. ***B***, Normalized NADH peak fluorescence (*F*) values of mechanically stimulated astrocytes. When treated with CGP37157, FCCP, and oligomycin, astrocytes had significantly lower peak fluorescence than control. Astrocytes treated with Ru360 had significantly higher peak fluorescence than control. CsA had not significantly affected the peak values of mechanically induced glutamate release from astrocytes. ***C***, Normalized NADH cumulative fluorescence (*F*) values obtained from mechanically stimulated astrocytes. Pharmacological agents had similar effects as in ***B***, with the exception of CsA that caused a decrease in the cumulative glutamate release. The asterisks denote significant change compared with control group (one-way ANOVA, followed by Fisher's LSD test, $^{**}p < 0.01$).

cantly increased or insignificant (although it showed a trend in increase), respectively (compare Figs. 2, 3).

Together, we demonstrated that Ru360 increased the peak and cumulative release of glutamate in astrocytes, as a result of its action in increasing Ca^{2+}_{cyt}. We also demonstrated that decreasing Ca^{2+}_{cyt} by blocking Ca^{2+} efflux from mitochondria through the Na^+/Ca^{2+} exchanger using CGP37157, or enhancing mitochondrial Ca^{2+} accumulation with CsA, decreased cumulative glutamate release. It should be noted that CsA did not affect the peak glutamate release, however. Together, these data further support the role of the mitochondria in buffering increases in Ca^{2+}_{cyt} that govern exocytotic glutamate release from astrocytes. Interestingly, the reduction of the peak and cumulative glutamate release by FCCP, while causing increased Ca^{2+}_{cyt} responses, could arise from its ability to collapse vesicular proton gradient and thereby cause depletion of vesicular transmitter (Maycox et al., 1988; Takamori et al., 2000; Schäfer et al., 2002). Similarly, oligomycin, which only induced a marginal increase in peak Ca^{2+} response and did not affect the cumulative Ca^{2+} response, caused a decrease in glutamate release. These data suggest that the ATP/ADP ratio in astrocytes may play a role in glutamate release. This would be consistent with the observation that ATP is required for V-ATPase to generate the proton gradient that enables secretory vesicles to take up glutamate, and employ the mechanisms that dock/prime vesicles at the plasma membrane. We further tested these possible alternative sites of action by FCCP and oligomycin by monitoring intravesicular pH.

FCCP, but not oligomycin, dissipates vesicular H^+ gradient

Whereas treatment with FCCP increased the mechanically induced peak and cumulative Ca^{2+}_{cyt} responses in astrocytes when compared with those of control cells (Fig. 2), this protonophore caused a decrease in the consequential peak and cumulative glutamate release from astrocytes (Fig. 3). Previous studies have shown that FCCP dissipated the pH gradient across the lumen of vesicles necessary for transmitter transport into the vesicles (Maycox et al., 1988; Takamori et al., 2000; Schäfer et al., 2002). Thus, we investigated whether FCCP decreased glutamate release from astrocytes by decreasing the vesicular pH gradient. To address this issue, we used astrocytes transfected with synapto-pHluorin to test whether FCCP and the other agents can cause changes in the vesicular lumen pH (Sankaranarayanan et al., 2000). Blockade of V-ATPase using bafilomycin A1 can collapse the proton gradient leading to alkalinization of intravesicular lumen by its equilibration to the cytosolic pH. Consistent with our previous study (Montana et al., 2004), incubation of astrocytes with bafilomycin A1 (5 μM; 30 min) in normal external solution, pH 7.35, caused the increase in synapto-pHluorin fluorescence (Fig. 4*A*,*B*) (one-way ANOVA followed by Fisher's LSD test, $p < 0.01$), which displayed a punctate pattern consistent with the vesicular location of synapto-pHluorin. Similarly, astrocytes bathed in normal external solution treated with FCCP, but not with CGP37157, CsA, Ru360, or oligomycin, exhibited a significant increase in synapto-pHluorin fluorescence when compared with time-matched controls (Fig. 4*A*,*B*) (one-way ANOVA, followed by Fisher's LSD test, $p < 0.05$). To address the responsiveness of synapto-pHluorin to alkalinization, at the end of each experimental run we incubated astrocytes in high pH external solution, pH 8.5. Although astrocytes possess mechanisms to control intracellular pH, the treatment with high pH external solution obviously overpowers them, because all transfected astrocytes exhibited increases in synapto-pHluorin fluorescence (Fig. 4*A*,*C*) because of alkalinization of the cytosol and consequential equilibration of vesicular pH to it (one-way ANOVA followed by Tukey–Kramer's procedure, $p < 0.01$) (Fig. 4, compare *B*, *C*); a punctate pattern of fluorescence was observed. It is, however, important to note that only astrocytes treated with bafilomycin A1 and placed in high pH external solution exhibited a significant augmentation of fluorescence when compared with the increased synapto-pHluorin fluorescence levels in control astrocytes placed in high pH external solution (Fig. 4*C*) (one-way ANOVA followed by Fisher's LSD test, $p < 0.01$). These findings are consistent with the notion that FCCP alkalin-

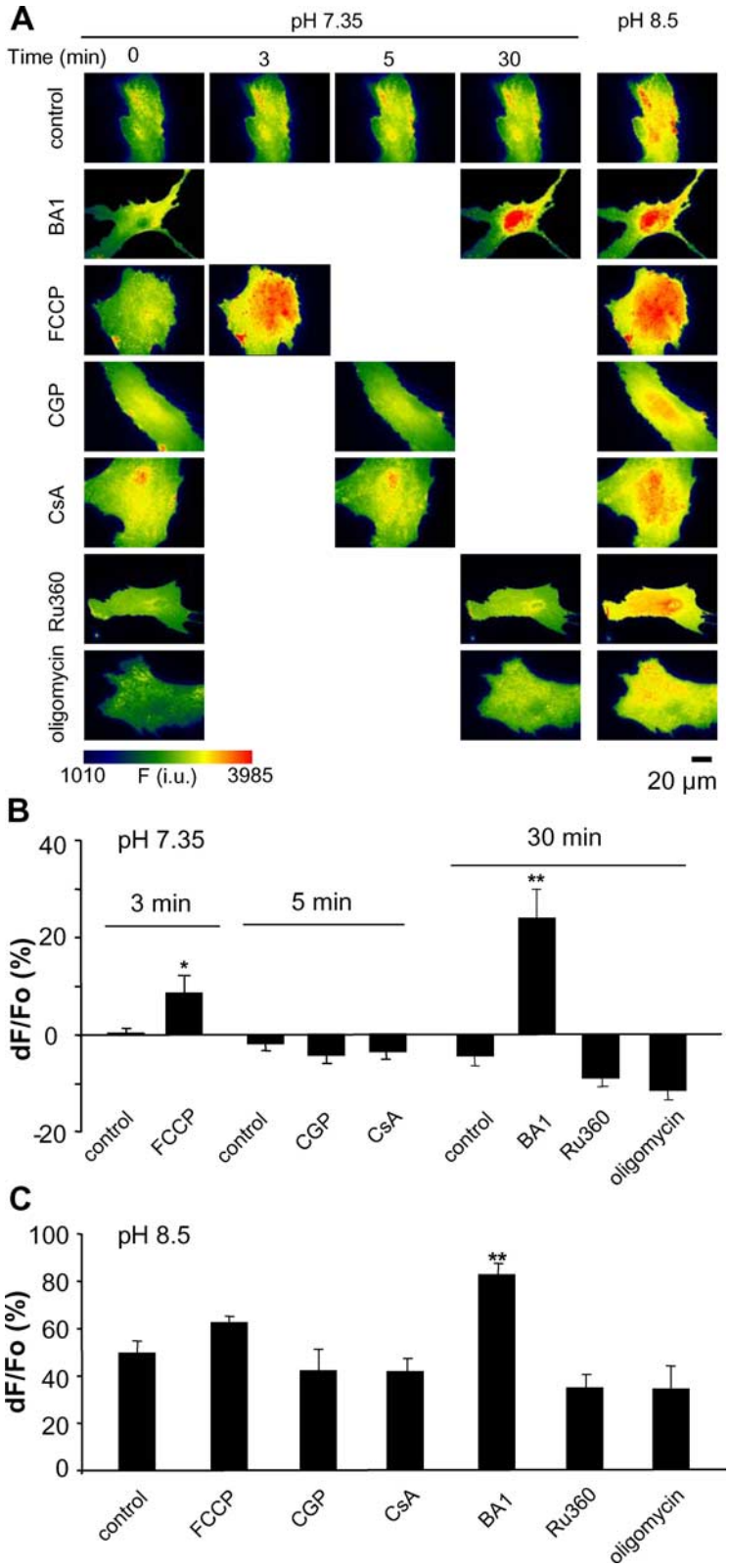

Figure 4. Effect of pharmacological agents on the luminal pH of secretory vesicles. Expression of synapto-pHluorin at vesicular locations leads to its fluorescence quenching by the acidity of the vesicular lumen, pH ~5.5. ***A***, Pseudocolored images (raw data) of the synapto-pHluorin fluorescence in sham-run (control) astrocytes, and in astrocytes treated with bafilomycin A1 (BA1) (5 μM; 30 min), and pharmacological agents preferentially affecting mitochondria: FCCP, CGP37157 (CGP), CsA, Ru360, or oligomycin. Addition of bafilomycin A1 to the astrocytes at rest expressing synapto-pHluorin (0 min) causes an increase in pHluorin fluorescence (30 min). Similarly, FCCP caused an increase in synapto-pHluorin fluorescence intensity compared with the time-matched control (3 min). The pseudocolor scale is a linear representation of the fluorescence intensities (*F*) ranging from 1010 to 3985 intensity units (i.u.). Scale bar, 20 μm. ***B***, Bar graph depicting analysis of synapto-pHluorin fluorescence in astrocytes bathed in normal external solution at pH 7.35. Astrocytes treated with bafilomycin A1 and FCCP had significant increase in fluorescence intensity when compared with the sham-run controls (one-way ANOVA, followed by Fisher's LSD test, $^{*}p < 0.05$, $^{**}p < 0.01$). ***C***, Synapto-pHluorin fluorescence in astrocytes bathed in high pH external solution, pH 8.5. At external pH 8.5, astrocytes pretreated with bafilomycin A1 showed significant augmentation in synapto-pHluorin fluorescence intensity when compared with the sham-run control. This increase was also significantly higher than the increase in synapto-pHluorin fluorescence in astrocytes at external pH 8.5 pretreated with FCCP. The asterisks denote significant change compared with control and FCCP-treated group (one-way ANOVA, followed by Fisher's LSD test, $^{**}p < 0.01$). Changes in synapto-pHluorin fluorescence (***B***, ***C***) are expressed as dF/F_o (percentage) after background subtraction. The bars represent means and SEMs; each group contains measurements from six to seven astrocytes.

izes the secretory vesicles by forming H^+ channels, which allow leakage of H^+ to the cytosol, thus shunting protons that are being pumped into vesicle by V-ATPase. This leads to unquenching of synapto-pHluorin fluorescence that should be lesser in magnitude than when V-ATPase is blocked. Indeed, bafilomycin A1, which disrupts active acidification of vesicles, allowed greater unquenching of synapto-pHluorin than FCCP (Fig. 4*C*) (one-way ANOVA followed by Fisher's LSD test, $p < 0.01$).

Together, the FCCP effect on enhancing Ca^{2+} response, which leads to a decreased glutamate release, is attributable to depletion of this transmitter from vesicles that undergo exocytosis. Because oligomycin does not increase synapto-pHluorin fluorescence, its inhibitory effect on glutamate release most likely is not related to defueling of V-ATPase, but rather it could be related to the requirement for ATP in vesicular docking/priming at the plasma membrane.

Discussion

Two aspects of the cytosolic Ca^{2+} kinetics in astrocytes attributable to mechanical stimulation were measured and analyzed: the peak and cumulative Ca^{2+} responses. The peak response represents the maximum Ca^{2+}_{cyt} in the stimulated astrocyte as a result of Ca^{2+} entry into the cytosol from ER stores and/or extracellular space, and immediate sequestration by mitochondria (Fig. 5). The cumulative response additionally represents the declining Ca^{2+}_{cyt}, as free Ca^{2+} is slowly released by mitochondria, and is removed by pumps, such as the plasma membrane Ca^{2+}-ATPase and smooth ER Ca^{2+}-ATPase, from the cytosol (Kim et al., 2005). A multiagent approach was taken to enhance and inhibit mitochondrial Ca^{2+}

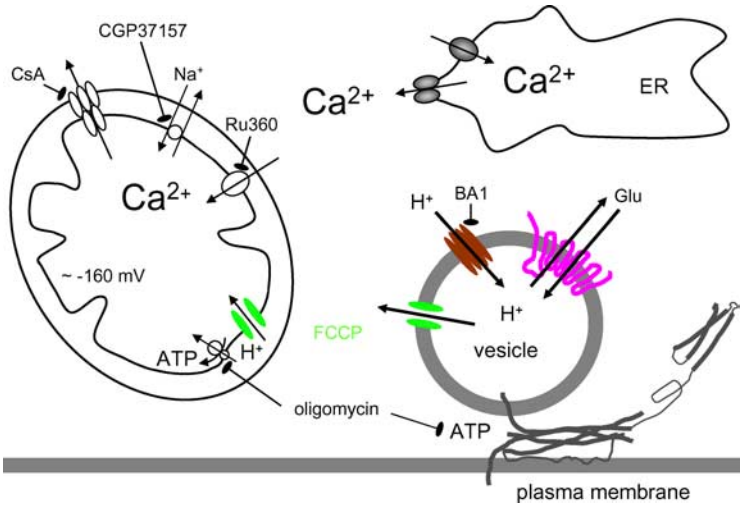

Figure 5. Dual Ca^{2+}_{cyt} handling in Ca^{2+}-dependent vesicular glutamate release from astrocytes. Ca^{2+}_{cyt} can be affected by the ER (see details in text) and mitochondria. A negative membrane potential (about −160 mV) exists across the inner mitochondrial membrane as a result of proton transport by the electron transport chain into the intermembrane space. Mitochondrial Ca^{2+} uptake is mediated by the uniporter as the electropotential gradient drives Ca^{2+} into the matrix. Ru360 blocks Ca^{2+} influx through the uniporter, whereas the protonophore, FCCP (green), dissipates the electrochemical gradient built up by proton transport, thus lowering the electropotential gradient for Ca^{2+} accumulation. ATP is produced by ATP synthase coupled to proton flux, a process blocked by oligomycin. Free Ca^{2+} exits the mitochondrial matrix through the Na^+/Ca^{2+} exchanger, and this process can be blocked by CGP37157. Ca^{2+} accumulation in mitochondria is increased by CsA via preventing formation of the mitochondrial permeability transition pore, which releases Ca^{2+} and other components of the mitochondrial matrix at high Ca^{2+} loads. Increase of Ca^{2+}_{cyt} is sufficient and necessary to cause vesicular fusions and release of glutamate, which requires action by proteins of the ternary SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) complex (dark gray). Additionally, vesicles possess the vacuolar type H^+-ATPase (V-ATPase; brown) and vesicular glutamate transporters (pink), which are necessary for acidification of vesicles and filling these organelles with glutamate (Glu), respectively. Bafilomycin A1 (BA1) can collapse the proton gradient by inhibition of V-ATPase. FCCP (green) alkalinizes secretory vesicles by forming H^+ channels, which allow leakage of H^+ to the cytosol. Oligomycin could deplete a fraction of the ATP reserves necessary for vesicle retention at release sites. This drawing is not to scale.

accumulation. This was done in part because some agents have multiple targets.

Consistent with previous studies in various neural cells, the present work provides evidence that mitochondria in solitary cortical astrocytes buffer Ca^{2+}_{cyt} elevations (Fig. 5). The Ca^{2+}_{cyt} response of agent-treated astrocytes was significantly greater than control astrocytes when the electrochemical gradient at the inner mitochondrial membrane was dissipated with FCCP (Thayer and Miller, 1990; Kiedrowski and Costa, 1995; White and Reynolds, 1995) or when the Ca^{2+} uniporter was blocked by Ru360 (Matlib et al., 1998). In contrast, the cytosolic Ca^{2+} response of agent-treated astrocytes was significantly reduced when the efflux of Ca^{2+} from mitochondria to the cytosol was blocked with the mitochondrial Na^+/Ca^{2+} exchanger inhibitor, CGP37157 (Baron and Thayer, 1997) or when the mitochondrial Ca^{2+} load was enhanced by CsA (Bambrick et al., 2006). Oligomycin, which inhibits ATP synthase, had a dual effect on the Ca^{2+} response. It significantly increased the peak Ca^{2+} response but did not affect cumulative Ca^{2+} response. This observation may suggest that oligomycin inhibited mitochondrial Ca^{2+} accumulation, but had little or no effect on Ca^{2+} clearance.

Previous studies provide evidence that mitochondrial Ca^{2+} buffering modulates exocytosis. In rat pituitary gonadotrophes (Kaftan et al., 2000), and in bovine chromaffin cells (Giovannucci et al., 1999), increasing Ca^{2+}_{cyt} concentration through suppression of mitochondrial Ca^{2+} accumulation, increased vesicle fusions as measured by an increase in membrane capacitance. It has been suggested that mitochondria limit the diffusion range of free Ca^{2+} and hence prevent additional vesicles in the readily releasable pool to fuse. In the present study, the relationship between mitochondrial Ca^{2+} buffering and glutamate released from astrocytes was drawn (Fig. 5). The present study demonstrated a correlation between elevated cytosolic Ca^{2+} transients, as mitochondrial Ca^{2+} accumulation was inhibited, and larger glutamate release. Ru360-treated astrocytes exhibited greater Ca^{2+} response as well as greater glutamate release compared with controls. In contrast, CGP37157- and CsA-treated astrocytes exhibited decreased Ca^{2+} response as well as decreased glutamate release compared with untreated astrocytes. Interestingly, FCCP-treated astrocytes show decreased glutamate release, although these cells exhibited greater Ca^{2+} response compared with untreated astrocytes. Using synapto-pHluorin as a reporter of vesicle pH, we demonstrated that FCCP alkalinized the vesicles, thereby decreasing the amount of glutamate packed into vesicles leading to reduced glutamate release. Unlike bafilomycin A1, which inhibits the V-ATPases, it appears that FCCP caused H^+ leakage by intercalating into the vesicle membrane (Fig. 5), consistent with previous findings (Takamori et al., 2000; Schäfer et al., 2002). Furthermore, it should be noted that oligomycin, which inhibits ATP synthase, also decreased glutamate release. Global intracellular ATP depletion, however, may be an unlikely explanation for this action because Ru360, which is known to decrease ATP levels (Wu et al., 2007), did not decrease glutamate release in solitary astrocytes. A possible explanation is that oligomycin specifically depleted a fraction of the ATP reserves necessary for vesicle retention at release sites (Fig. 5).

The observation that mitochondrial Ca^{2+} buffering can modulate glutamate release in astrocytes is important in light of studies showing that mitochondria act as a buffer at physiological Ca^{2+} concentrations in the CNS. Mitochondria of presynaptic neurons are critical Ca^{2+} buffers during slow release of free Ca^{2+} that enhances synaptic transmission in the span of minutes via a process referred to as posttetanic potentiation (Tang and Zucker, 1997). In addition, presynaptic mitochondria may modulate synaptic transmission by accelerating recovery from synaptic depression (Billups and Forsythe, 2002). How mitochondria shape Ca^{2+} dynamics and regulate glutamate release in astrocytes *in situ* is unknown and may be a key in understanding the tripartite synapse. In neurons, vesicular fusions occur at Ca^{2+} microdomains, areas of the synapse that experience rapid Ca^{2+} flux and that are enriched with voltage-gated and ligand-gated Ca^{2+} channels (Llinás et al., 1995). Although the precise locations of astrocytic exocytotic fusion sites are not readily apparent at present, it should be noted that mitochondria are highly enriched at the astrocytic processes that encompass synapses (Lovatt et al., 2007). It is then tempting to speculate that a subset of mitochondria may associate with astrocytic release sites to modulate Ca^{2+} dynamics at their putative Ca^{2+} microdomains. An interesting observation in our study was that FCCP and oligomycin caused fragmentation of mitochondria as visualized with 4-Di-2-ASP.

Therefore, it is possible that some of the decrease in glutamate release that occurred with these agents might be caused in part by modulation of Ca^{2+} dynamics at putative astrocytic Ca^{2+} microdomains.

Although mitochondria are an important buffer for pathological Ca^{2+} concentrations induced by glutamate toxicity in neurons (Wang and Thayer, 1996; Kannurpatti et al., 2000; Baron et al., 2003), the role of astrocytic mitochondria in buffering pathological Ca^{2+} concentrations is not well understood. Recent evidence suggests that there may be fundamental differences in Ca^{2+} buffering and metabolism between astrocytic and neuronal mitochondria (Bambrick et al., 2006; Lovatt et al., 2007). The observation that mitochondrial Ca^{2+} buffering may regulate glutamate release in astrocytes in the present study is important because astrocytic glutamate can be a source of neuronal excitation that underlies epilepsy (Tian et al., 2005; Ding et al., 2007). It is then possible that dysregulation of astrocytic mitochondrial Ca^{2+} buffering may lead to changes in regulated glutamate release from astrocytes.

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