Properties of Ca$^{2+}$-Dependent Exocytosis in Cultured Astrocytes

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ABSTRACT Astrocytes, a subtype of glial cells, have numerous characteristics that were previously considered exclusive for neurons. One of these characteristics is a cytosolic [Ca$^{2+}$] oscillation that controls the release of the chemical transmitter glutamate and atrial natriuretic peptide. These chemical messengers appear to be released from astrocytes via Ca$^{2+}$-dependent exocytosis. In the present study, patch-clamp membrane capacitance measurements were used to monitor changes in the membrane area of a single astrocyte, while the photolysis of caged calcium compounds by a UV flash was used to elicit steps in [Ca$^{2+}$]i to determine the exocytotic properties of astrocytes. Experiments show that astrocytes exhibit Ca$^{2+}$-dependent increases in membrane capacitance, with an apparent Kd value of $\sim$20 μM [Ca$^{2+}$]i. The delay between the flash delivery and the peak rate in membrane capacitance increase is in the range of tens to hundreds of milliseconds. The pretreatment of astrocytes by the tetanus neurotoxin, which specifically cleaves the neuronal/neuroendocrine type of SNARE protein syntaptobrevin, abolished flash-induced membrane capacitance increases, suggesting that Ca$^{2+}$-dependent membrane capacitance changes involve tetanus neurotoxin-sensitive SNARE-mediated vesicular exocytosis. Immunocytochemical experiments show distinct populations of vesicles containing glutamate and atrial natriuretic peptide in astrocytes. We conclude that the recorded Ca$^{2+}$-dependent changes in membrane capacitance represent regulated exocytosis from multiple types of vesicles, about 100 times slower than the exocytotic response in neurons.

INTRODUCTION Recent advances in astrocyte physiology showing that these glial cells may not only support neuronal networks, but may be key in modulating synaptic transmission by releasing chemical messengers such as ATP and glutamate (Jeftinija et al., 1996; Haydon, 2001), raised the question as to whether the mechanism of release of these chemical messengers involves regulated exocytosis, which requires an increase in cytosolic calcium as a trigger. Key experiments that led to the formulation of the “calcium hypothesis” were carried out about 50 years ago (Harvey and MacIntosh, 1940; Katz and Miledi, 1965; Dodge and Rahamimoff, 1967). This hypothesis holds that Ca$^{2+}$ binds to a component of the presynaptic structure to trigger the release of neurotransmitter. The introduction of electrophysiological membrane capacitance (Cm) measurements, a pa-
rameter linear to the membrane area (Neher and Marty, 1982), greatly advanced our understanding of regulated exocytosis. Initial studies on neuroendocrine cells (Neher and Marty, 1982; Siklar et al., 1989; Thomas et al., 1990; Zorec et al., 1991) and on neuronal cells (Heidelberger et al., 1994; Schneggenburger and Neher, 2000; Bollmann et al., 2000) revealed in detail the biophysical and biochemical properties of regulated exocytosis.

It has been shown that intracellular Ca\(^{2+}\) levels regulate the release of classical neurotransmitters such as glutamate from astrocytes (Parpura et al., 1994; Bezzi et al., 1998; Araque et al., 2000; Pasti et al., 2001), and that glutamate release correlates to surface area changes (Zhang et al., 2004). Astrocytes also possess several components of SNARE molecules (Parpura et al., 1995; Zhang et al., 2004); key elements for vesicular exocytosis (Geppert and Südhof, 1998) and subcellular organelles that may be involved in regulated exocytosis have been described (Calegari et al., 1999; Coco et al., 2003; Kržan et al., 2003), but direct evidence for Ca\(^{2+}\)-dependent membrane area increase due to SNARE-dependent vesicle exocytosis in astrocytes is not available.

Using membrane capacitance measurements and flash photolysis of caged calcium compounds (Neher and Zucker, 1993; Rupnik et al., 2000), we show that astrocytes exhibit Ca\(^{2+}\)-dependent membrane capacitance increases that are blocked by the pretreatment of cells by the tetaus toxin, suggesting that Ca\(^{2+}\)-dependent membrane capacitance increases represent neuron-like SNARE-associated vesicular fusion. Furthermore, immunocytochemical studies revealed distinct vesicular compartments containing glutamate and atrial natriuretic peptides, both thought to be released from astrocytes via regulated exocytosis (Haydon, 2001; Kržan et al., 2003). We conclude that Ca\(^{2+}\)-dependent increases in membrane capacitance represent an exocytotic response of astrocytes consisting of multiple vesicular exocytotic pathways. In comparison with the rapid kinetics of exocytotic response in neuronal cells assayed by similar measurements (Heidelberger et al., 1994; Parsons et al., 1994; Mennerick and Matthews, 1996; Gomis et al., 1999; Neves and Lagnado, 1999; Beutner et al., 2001; Kreft et al., 2003), the delay to the maximal rate of membrane capacitance increase in astrocytes suggests an ~2 orders of magnitude slower exocytotic response, consistent with the view that astrocytes play a role as integrators in glial-to-neuron and glia-to-endothelia signaling.

**MATERIALS AND METHODS**

**Materials**

Unless mentioned otherwise, all materials were obtained from Sigma Chemical Company (St. Louis, MO).

**Cell Culture and Electrophysiology**

Cultures were prepared from the cortex of 3-day-old rats as described (Schwartz and Wilson, 1992). Cells were grown in high-glucose Dulbecco’s modified Eagle’s medium (DMEM), containing 10% fetal bovine serum (FBS), 1 mM pyruvate, 2 mM glutamine, and 25 μg/ml penicillin/streptomycin in 95% air/5% CO\(_2\). Confluent cultures were shaken at 225 rpm overnight, and the medium was changed the next morning; this process was repeated a total of three times. After the third shaking, the cells were trypsinized and cultured for 24 h in 10 μM cytosine arabinoside. After reaching confluence again, the cells were subcultured onto 22-mm-diameter poly-L-lysine-coated coverslips. Compensated \(C_m\) measurements were used (Zorec et al., 1991) employing a SWAM IIB amplifier (Celica, Ljubljana, Slovenia), operating at 800-Hz lock-in frequency (sine wave of 111 mV rms). Upon establishment of the whole-cell configuration compensation with \(C_{slow}\) and \(G_a\) controls provided readings of 18.6 ± 1.6 pF and of 140.2 ± 12.6 nS (n = 23, mean ±SE), respectively, indicating a simple electrical geometry of nonspherical astrocytes. The phase angle setting was determined by a 1-pF calibration pulse and by monitoring the projection of this pulse from the out-of-phase signal C (proportional to \(C_m\)) to the in-phase signal G of the lock-in amplifier. Signals were stored unfiltered (C-DAT4 recorder, Cygnus, Fort Atkinson, WI) for off-line analysis. Simultaneously, we recorded filtered (300-Hz, 4 pole Bessel) C and G signals, and the fluorescence intensity from a C660 photon counter (Thorn EMI, Byfleet, UK). PhoCal program (LSR, Cambridge, UK) was used to acquire signals every 5 ms. For high temporal resolution measurements of \(C_m\), the records on DAT were played back and a 10-s epoch of the signal enveloping each flash was digitized at 50 kHz, using a CDR program (J. Dempster, Glasgow, UK). Signals were digitally filtered at 1 kHz (2-way 150th order FIR filter, Math Works MATLAB, Natick, MA) and resampled at 10 kHz. The pipette solution contained (in mM): KCl 110, TEACl 10, KOH/HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) 40, Na\(_2\)ATP 2, MgCl\(_2\) 2, K\(_4\)-NP-EGTA [o-nitrophenyl ethyleneglycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetrapotassium salt] 4, CaCl\(_2\) 3.6, furaptra 0.5, pH = 7.2. The bath contained (in mM): NaCl 131.8, KCl 5, MgCl\(_2\) 2, NaH\(_2\)PO\(_4\) 0.5, NaHCO\(_3\) 5, NaOH/HEPES 10, D-glucose 10, CaCl\(_2\) 1.8, pH = 7.2. All recordings were made at room temperature. Cells were voltage-clamped at a holding potential of ~50 mV. Recordings were made with pipette resistances between 1 and 4 MΩ (measured in KCl-rich solution). The pipette and bath solutions were of similar osmolarity (within 5%) measured by freezing point depression (Camlab, Cambridge, UK).

**Toxin Microinjection**

Transjector 4657 with a micromanipulator (Eppendorf, Hamburg, Germany) was used. Pulses (8–12 hPa, 0.3–1s) were applied with a compensation pressure (1 hPa). Microinjection solution consisted of (in mM): K-gluconate 150, MgCl\(_2\) 2, HEPES 10, RITC 6 mg/ml,
tetanus toxin (20 ng/μl, a gift from Dr. Das Gupta, Madison, WI). Pipettes were prepared with a horizontal puller (P-87, Sutter Instruments, Novato, CA). Microinjected cells were identified by co-injected rhodamine-labeled dextran (25 kDa). Before continuing with patch-clamp experiments, the cells were allowed to rest for 1–2 h at 37°C.

**Calcium Measurements and Flash Photolysis**

The Ca\(^{2+}\) cage NP-EGTA (Molecular Probes, Eugene, OR) was used to elevate \([\text{Ca}^{2+}]_i\) by flash photolysis (Ellis-Davies and Kaplan, 1994). A 1-ms ultraviolet (UV) flash from a Xenon arc flash lamp (Hi-Tech, Salisbury, UK) was delivered to cells through a 40× fluor oil immersion objective (NA = 1.3) of a Nikon Diaphot microscope (Rupnik et al., 2000). The same optical pathway as in flash photolysis was used to illuminate the \([\text{Ca}^{2+}]_i\) indicator furaptra (Molecular Probes, Eugene, OR). A combination of two dichroic mirrors was used. The first was a 390-nm dichroic positioned at 45°, which passed through the 420-nm light from a Xe arc lamp for furaptra excitation and reflected the light below 390 nm for NP-EGTA flash photolysis from a Xe arc flash lamp. The second mirror, a 430-nm dichroic, reflected both lights through the objective to the cell studied and allowed only furaptra fluorescent light to pass back to the photomultiplier through a 510-nm barrier filter. \([\text{Ca}^{2+}]_i\) measurements were performed as described (Rupnik et al., 2000), using the equation:

\[
[\text{Ca}^{2+}]_i = K_d \frac{F - F_{\text{min}}}{F_{\text{max}} - F}
\]

where \(F_{\text{max}}\) is the autofluorescence in the cell-attached configuration, \(F_{\text{min}}\) is fluorescence in a resting whole-cell recording, and \(F\) is fluorescence during the flash. \(K_d\) for furaptra is 25 μM.

**Lipofection, Immunocytochemistry, and Confocal Microscopy**

The plasmid encoding atrial natriuretic factor, tagged with emerald green fluorescent protein, ANP.emd (Han et al., 1999), was introduced into the astrocytes by lipofection, using the Invitrogen (Carlsbad, CA) protocol. DNA was mixed with 6 μl Plus Reagent, diluted in 100 μl serum-free DMEM, and was incubated for 15 min at room temperature (RT). In this study, 4 μl lipofectamine™ was diluted in 100 μl serum-free DMEM. After incubation, both solutions were mixed and incubated further for 15 min at RT. Meanwhile, astrocytes were washed once with serum-free DMEM and supplemented with 800 μl DMEM; 200 μl of lipofection mixture was pipetted onto the cells, which were further incubated for 3 h at 95% air/5% CO\(_2\) at 37°C. Then 30 μl of Ultroser G (Gibco, Grand Island, NY) was added, and DMEM was exchanged the next day. Astrocytes expressing ANP.emd were washed once with 1 ml of phosphate-buffered saline (PBS) and fixed in paraformaldehyde (4% in PBS) for 15 min. Cells were permeabilized with 4% paraformaldehyde, 0.1% Triton X-100 for 10 min and washed 4× with PBS. Subsequently, a rabbit serum containing either anti-VGLUT1 (1:1,000) or anti-VGLUT2 (1:1,000) antibody was applied to the astrocytes, which were further incubated overnight at 4°C. The cells were then washed 4× with PBS and exposed to the respective secondary antibody solution, an Alexa Fluor 546-conjugated anti-rabbit IgG (1:500). Cells were washed 4× with PBS and preparation finally supplied with Slow Fade Light Anti-fade Kit (Molecular Probes, Leiden, Netherlands). Coverslips with double-labeled astrocytes were examined with a Zeiss confocal microscope (LSM 510, Jena, Germany). Fluorescent images were acquired by a plan-apochromatic oil immersion objective (63× magnification, 1.4 NA) using 488-nm Ar-Ion and 543-nm He-Ne laser excitation. ANP.emd fluorescence and fluorescence of labeled VGLUT1 and VGLUT2 were separated using BP 505–530-nm and LP 560-nm emission filters, respectively. Images were stored on IBM-PC compatible computer (Siemens Nixdorf, Germany). To quantify images, these were exported into TIFF files and analyzed with a custom-made MATLAB program (Kreft et al., 2004). Briefly, the program counted all red, all green, and all colocalized pixels in the image. The degree of colocalization between green ANP.emd and fluorescently labeled antibodies against ANP, VGLUT1 and V-GLUT2 in red, was expressed as percentage of colocalized pixels in comparison to all green pixels. Colocalization data were statistically analyzed using unpaired, two-tailed Student’s t-test.

**RESULTS**

Exocytosis involves the addition of membrane to the plasma membrane, while endocytosis involves subtraction of membrane; thus, both processes contribute to membrane area fluctuations (Neher and Marty, 1982). If regulated Ca\(^{2+}\)-triggered exocytosis exists in astrocytes, an increase in \([\text{Ca}^{2+}]_i\) should elicit an increase in \(C_m\), a parameter that is linearly proportional to the membrane area (Neher and Marty, 1982). We monitored secretory activity using compensated \(C_m\) measurements (Rupnik et al., 2000) combined with flash photolysis to deliver rapid and spatially homogeneous steps in cytosolic \([\text{Ca}^{2+}]_i\), (Neher and Zucker, 1993). The calcium cage NP-EGTA, preloaded with Ca\(^{2+}\), was introduced into the cytosol of single astrocytes using patch pipette dialysis. Single astrocytes, devoid of contacts with neighboring cells, were used to avoid the potential problems of electric non-homogeneity due to gap junction coupling between cells. UV flash application (arrows) transiently elevated \([\text{Ca}^{2+}]_i\) to a peak value, which subsequently returned exponentially to the baseline with a time constant of 2–5 s (Fig. 1, top.
A change in C<sub>m</sub> was observed. Moreover, repeated flash with different UV flash intensities. Peak [Ca<sup>2+</sup>][i] elicited a large rise in C<sub>m</sub>, which persisted at elevated level after the [Ca<sup>2+</sup>][i] returned to basal level (Fig. 1A, bottom trace). Second, the peak in C<sub>m</sub> was followed by a delayed decrease in C<sub>m</sub>, dominated by endocytosis. The extent of endocytosis was determined by measuring the decrease in C<sub>m</sub> 1 min after the flash (endo). The resting capacitance of this cell was 17 pF.

Figure 1 (bottom traces) shows two types of Ca<sup>2+</sup>-induced membrane capacitance (C<sub>m</sub>) increase in astrocytes. Time-dependent changes in C<sub>m</sub> in a cultured astrocyte. A: Following the UV flash induced peak in C<sub>m</sub>, the amplitude persisted at an elevated level. Resting capacitance of this cell was 29 pF. B: In cells exposed to higher cytosolic [Ca<sup>2+</sup>][i], the peak in C<sub>m</sub> was followed by a delayed decrease in C<sub>m</sub>, dominated by endocytosis. The extent of endocytosis was determined by measuring the decrease in C<sub>m</sub> 1 min after the flash (endo). The resting capacitance of this cell was 17 pF.

traces). Figure 1 (bottom traces) shows two types of time-dependent changes in C<sub>m</sub> in astrocytes. First, increased [Ca<sup>2+</sup>][i], elicited a large rise in C<sub>m</sub>, which persisted at elevated level after the [Ca<sup>2+</sup>][i] returned to basal level (Fig. 1A, bottom trace). Second, the peak in C<sub>m</sub> was followed by a delayed decrease in C<sub>m</sub>, caused by endocytotic retrieval of membrane (Fig. 1B). Endocytosis was evaluated by measuring the decrease in C<sub>m</sub> 1 min after the flash. With the peak [Ca<sup>2+</sup>][i] between 10 and 20 μM, endocytosis was detected in 5 of 15 responses with an average decrease of 0.2 ± 0.1 pF (mean ± standard error, N = 5). With a peak post-flash [Ca<sup>2+</sup>][i] of 20–60 μM, endocytosis was present in 12 of 14 responses with an average decrease in C<sub>m</sub> of 2.8 ± 0.9 pF (mean ± standard error, N = 12). From this one can estimate the average rate of endocytosis to be around 50 fF/s. These experiments also indicate that the occurrence of endocytosis is dependent on the level of stimulation (Artalejo et al., 1995; Engisch and Nosycky, 1998). It is unlikely that these responses are due to an artifact, since when the NP-EGTA in the pipette lacked Ca<sup>2+</sup>, a UV flash failed to elevate [Ca<sup>2+</sup>][i], and no change in C<sub>m</sub> was observed. Moreover, repeated flash applications resulted in repeated increases in C<sub>m</sub> (not shown), suggesting that flash applications did not result in phototoxicity.

At a peak [Ca<sup>2+</sup>][i] of 15 μM, the amplitude of the flash-induced C<sub>m</sub> response was 700 fF (Fig. 1A), whereas it increased to 1,750 fF at 28 μM [Ca<sup>2+</sup>][i] (Fig. 1B), indicating a Ca<sup>2+</sup>-dependent addition of membrane due to exocytosis. At higher time resolution, three responses in C<sub>m</sub> are shown in Figure 2A, each recorded at a different [Ca<sup>2+</sup>][i] obtained by photolysis with different UV flash intensities. Peak [Ca<sup>2+</sup>][i] (in μM) is indicated near each of the traces. By increasing the [Ca<sup>2+</sup>][i], beyond 10 μM we were able to reliably elicit a detectable increase in C<sub>m</sub> (Figs. 1 and 2A). Not only the amplitude of responses, but also the rate of C<sub>m</sub> increase, a measure of the rate of exocytosis, depended in a sigmoidal fashion on [Ca<sup>2+</sup>][i] (Fig. 2C). The fitting of a curve (Fig. 2C) suggests that ~20 μM [Ca<sup>2+</sup>][i] is required for the half-maximal response in C<sub>m</sub>.

The delay between UV flash delivery and the peak rate of C<sub>m</sub> increase was reduced as [Ca<sup>2+</sup>][i] increased (Fig. 2B,D), consistent with the Ca<sup>2+</sup> requirement for the triggering of exocytosis in astrocytes. It is unlikely that the very slow, stimulation-dependent endocytosis...
(Fig. 1B; ~50 fF/s) greatly affects the initial maximal rate of $C_m$ increase of 3,000 fF/s. Thus, the flash-induced increase in $C_m$ is determined mainly by Ca$^{2+}$-regulated exocytosis.

To establish further that Ca$^{2+}$-induced changes in $C_m$ are due to regulated vesicular exocytosis, we tested whether Ca$^{2+}$-induced changes in $C_m$ are sensitive to neurotoxins that specifically cleave the SNARE proteins, essential elements of neuronal/neuroendocrine vesicle exocytosis (Montecucco and Schiavo, 1994), but not those associated with the endosomal and lysosomal subcellular compartments (Martinez-Arca et al., 2000). Figure 3A shows a time-dependent change in [Ca$^{2+}$]$\text{i}$ and in $C_m$, both in a control and in a neurotoxin-treated cell. The maximal rate of $C_m$ increase was strongly inhibited by tetanus toxin pretreatment (Fig. 3B), supporting the view that Ca$^{2+}$-dependent changes in $C_m$ consist mainly of tetanus toxin-sensitive vesicular exocytosis. The small residual tetanus toxin-insensitive Ca$^{2+}$-dependent changes in $C_m$ (Fig. 3B) suggest that tetanus toxin-insensitive vesicle exocytosis (Martinez-Arca et al., 2000) appears to be very small or absent in cultured astrocytes.

The release of both glutamate (Parpura et al., 1994) and atrial natriuretic peptide (ANP; Kržan et al., 2003) requires Ca$^{2+}$. While it is generally believed that small molecular weight neurotransmitters and peptides segregate into distinct vesicles, some experiments suggest that glutamate and peptide may coexist in a single vesicle (Maechler and Wollheim, 1999). We used an immunocytochemical approach to study the subcellular distribution of ANP and markers for glutamate-containing vesicles, such as the vesicle glutamate transporters VGLUT1 and VGLUT2 (Bellochio et al., 2000; Takamori et al., 2000). Figure 4 shows a punctuate appearance for the fluorescence signals of both markers, consistent with the notion that both markers are localized to storage organelles such as vesicles. Furthermore, there appears to be no overlap between the fluorescence signals, indicating the coexistence of distinct subcellular storage organelles for peptides and for glutamate in astrocytes. To quantify further whether the two markers are colocalized, we measured the level of colocalization between ANP.emd fluorescence and the fluorescence from the anti-ANP, anti-VGLUT1 and anti-VGLUT2 antibodies, respectively. In controls, the colocalization between ANP.emd and immunolabeled anti-ANP antibody was 81 ± 10% (mean ± SE), significantly higher with respect to the colocalization between ANP.emd and either anti-VGLUT1 (13 ± 2%) or anti-VGLUT2 (13 ± 3%) antibodies, respectively (Fig. 5), indicating that ANP and vesicular glutamate transporters reside in distinct subcellular organelles in cultured astrocytes.

These data indicate that distinct types of vesicles may contribute to the recorded flash-induced responses in $C_m$, with the presence of multiple exocytotic pathways in astrocytes, as has been found in a number of other cell types (Kasai, 1999; Rupnik et al., 2000; Voets et al., 2001; Poberaj et al., 2002).

**DISCUSSION**

Although a large number of substances are reported to be secreted from astrocytes (Martin, 1992; Parpura et al., 1994), the underlying mechanisms are still debated. In the case of the transmitter glutamate secretion, for example, reversal of glutamate transporters, anion transporter-dependent mechanisms, channel permeation, as well as Ca$^{2+}$-dependent exocytosis have all been proposed (Attwell, 1994; Haydon, 2001; Duan et al., 2003). In support of the latter hypothesis,
it was shown recently that membrane surface area changes and optically detected glutamate release are associated, supporting the notion that glutamate is released by exocytosis (Zhang et al., ).

The aim of this study was to determine the properties of Ca\(^{2+}\)-dependent exocytosis in astrocytes by using electrophysiological measurements of membrane capacitance. Exocytosis involves addition of membrane to the plasma membrane, while endocytosis involves subtraction of membrane; thus, both processes actively contribute to membrane area fluctuations (Neher and Marty, 1982). If regulated Ca\(^{2+}\)-triggered exocytosis exists in astrocytes, an increase in [Ca\(^{2+}\)]\(_i\) should elicit an increase in C\(_m\), a parameter linearly proportional to the membrane area (Neher and Marty, 1982). Consistent with the key involvement of Ca\(^{2+}\) in regulated
exocytosis pathway flash photolysis of NP-EGTA, a calcium cage compound, elicited an increase in [Ca$^{2+}$], and a consequent rapid rise in $C_m$. The half-maximal response was obtained at ~20 μM [Ca$^{2+}$], (Fig. 2C). This concentration may be exceeded under physiological conditions (i.e., close to the mouth of open IP3 receptors that mediate calcium flux from the internal stores to the cytosol). The relatively high Hill’s coefficient of 3.3 (see the equation in Fig. 2C) suggests that at least five calcium ions must bind to a calcium sensor to activate vesicle fusion or alternatively that there are distinct mechanisms for calcium sensing by vesicles that respond to calcium. Therefore, cultured astrocytes appear to possess the machinery for regulated exocytosis.

It was shown previously that glutamate is released from astrocytes at submicromolar cytosolic [Ca$^{2+}$] (Parpura and Haydon, 2000). There are three possible reasons for the apparent discrepancy between the apparent $K_d$ of 20 μM reported in this study and the previous work. First, the previous study only demonstrated that submicromolar elevations of [Ca$^{2+}$] were sufficient to evoke transmitter release. Because a full dose-response relationship between [Ca$^{2+}$] and glutamate release was not determined, the $K_d$ for [Ca$^{2+}$] in the previous study remains unknown. For example, it is possible that the previous study only operated at the foot of the sigmoid relationship between [Ca$^{2+}$] and release, which would explain why low [Ca$^{2+}$] could stimulate release and why a Hill coefficient of only 2 was measured, compared with 5 in the current study. Second, regulated exocytosis may be of different modes. The kiss-and-run fusion may result in a relatively small net increase in membrane capacitance increase in comparison to complete fusion where the vesicle membrane is incorporated into the plasma membrane. In the kiss-and-run mode of exocytosis, small-molecular-weight molecules (e.g., glutamate) will escape the vesicle lumen during relatively short (ms) transient openings of the fusion pore. If glutamate release were mediated predominantly by kiss-and-run fusion, this contribution to the whole-cell capacitance response measured in this case would be relatively small compared with the total membrane insertion measured. A third possibility is that because capacitance measurements do not discriminate between different vesicle pools with different $K_d$ values the abundance of glutamate containing vesicles might be small in comparison with all vesicles that contribute to the regulated exocytosis in astrocytes.

Other secretory vesicles, such as lysosomes, may contribute to changes in $C_m$ as well. Secretory lysosomes exocytose in a Ca$^{2+}$-dependent manner (Martinez et al., 2000), and it is possible that the Ca$^{2+}$-dependent changes in membrane capacitance records in astrocytes and fibroblasts (Ninomiya et al., 1996; Coorssen et al., 1996) represent, at least in part, a contribution of Ca$^{2+}$-dependent lysosomal exocytosis required for membrane wound repair (Reddy et al., 2001). Consistent with this, it was found that synaptotagmin VII, a protein required for Ca$^{2+}$-dependent lysosome exocytosis (Martinez et al., 2000) was reported to be expressed in astrocytes (Zhang et al., 2003). However, lysosomal membranes contain VAMP 7 (Advani et al., 1999), a SNARE protein that is insensitive to tetanus toxin (Martinez-Arca et al., 2000). Therefore, it is likely that fusion of lysosome membrane with other membranes is insensitive to this neurotoxin. The small residual, tetanus toxin-insensitive flash induced $C_m$ increase (Fig. 3B) suggests that, in astrocytes, the tetanus toxin-insensitive vesicle exocytosis is small in comparison with the tetanus toxin-sensitive vesicle exocytosis. This component also shares the Ca$^{2+}$-sensitivity of regulated exocytosis in various neuronal preparations (Heidelberger et al., 1994; Schneggenburger and Neher, 2000; Bollmann et al., 2000; Kreft et al., 2003). Therefore, the recorded Ca$^{2+}$-dependent increases in $C_m$ represent mainly thefusion of vesicles characteristic of neurons and neuroendocrine cells. The nature and the size of these vesicles are unknown and remain to be investigated in the future.

Although stimulation-dependent endocytosis is present in astrocytes (Fig. 1B), it is unlikely to have a significant influence on the flash-induced rate of exocytosis. The average rate of decrease in $C_m$ of 50 fF/s (see Fig. 1B) indicates that the rate of endocytosis in these cells is rather slow in comparison to the maximal rate of $C_m$ increase of 3,000 fF/s (Fig. 2C). To convert the rate of $C_m$ increase (Fig. 2B) into rate constants, we divided the maximal rates of $C_m$ increase by 1,000 fF, the average amplitude of the $C_m$ response when [Ca$^{2+}$]$_i$ was 20μM. At high [Ca$^{2+}$]$_i$, the rate constant reached a limiting value of 3/s (time-constant of 333 ms). In contrast, the measurements of the delay between the flash delivery and the maximal rate of $C_m$ increase revealed a limiting minimal delay of 50–100 ms (Fig. 2C, D). Both measurements show that, in cultured astrocytes, the kinetics of the Ca$^{2+}$-regulated exocytotic apparatus is similar to the exocytotic response in neuroendocrine cells (Rupnik et al., 2000; chromaffin cell), but that it is ~2 orders of magnitude slower in comparison with the rapid kinetic component of exocytosis in neuronal cells studied by similar techniques (Heidelberger et al., 1994; Parsons et al., 1994; Mennerick and Matthews, 1996; Gomis et al., 1999; Neves and Lagnado, 1999; Beutner et al., 2001, Kreft et al., 2003), and the delay in synaptic transmission (Sabatini and Regehr, 1999; Taschenberger and von Gersdorff, 2000). The relatively slow rate of exocytosis in astrocytes is probably related to structural differences between astrocytes and neurons. Rapid initial rates of exocytosis can be achieved in neurons because many vesicles are docked with the presynaptic plasma membrane in preparation for vesicle fusion and the release of neurotransmitter. Additionally, it was recently suggested that adjacent vesicles can fuse with one another through compound exocytosis to facilitate high rates of release (Parsons and Sterling, 2003). Because astrocytes do not have vesicles arranged in distinct clusters that are pre- docked with the plasma membrane, it is
perhaps not surprising that exocytosis is so slow compared with neurons. This property is consistent with the integrating function of astrocytes in neuron-to-glial and glia-to-endothelia signaling (Haydon, 2001; Kržan et al., 2003).

Glial cells exhibit a special form of excitability consisting of [Ca2+] oscillation, which may propagate between neighboring cells (Verkhratsky et al., 2002). Experimental evidence that neurotoxin sensitive Ca2+-dependent exocytosis is present in astrocytes, and that this process has a similar Ca2+-sensitivity to that found in neurons, suggests a new perspective on the role of astrocytes in the central nervous system. As in neurons, exocytosis plays a role in astrocytes in the release of chemical messengers such as ATP and glutamate, which lead to a modulation of synaptic transmission (Haydon, 2001). Moreover, exocytosis is involved in the release of peptides (Kržan et al., 2003), playing a role in the regulation of blood perfusion of the brain tissue. The kinetics of glial Ca2+-regulated exocytosis is slow in comparison with synaptic transmission (Sabatini and Regehr, 1999) (Figs. 1 and 2) and provides Ca2+-excitable astrocytes with the cellular machinery necessary for the integration and coordination of neurons as well as endothelia during CNS function.

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