Adenosine Triphosphate and the Late Steps in Calcium-dependent Exocytosis at a Ribbon Synapse

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ABSTRACT The ATP dependence of the kinetics of Ca^{2+}-dependent exocytosis after flash photolysis of caged Ca^{2+} was studied by capacitance measurements with submillisecond resolution in single synaptic terminals of retinal bipolar neurons. After control experiments verified that this combination of techniques is valid for the study of exocytosis in synaptic terminals, a comparison was made between the Ca^{2+} dependence of the rate of exocytosis in synaptic terminals internally dialyzed with MgATP, MgATP-γ-S, or no added Mg^{2+} or nucleotide. The Ca^{2+} threshold for release, the maximum rate of release, and the overall relationship between the rate of synaptic vesicle fusion and $[Ca^{2+}]_i$ were found to be independent of MgATP. A decrease in the average rate at near-threshold $[Ca^{2+}]_i$ was observed in terminals with MgATP-γ-S, but due to the small sample size is of unclear significance. The Ca^{2+} dependence of the delay between the elevation of $[Ca^{2+}]_i$ and the beginning of the capacitance rise was also found to be independent of MgATP. In contrast, MgATP had a marked effect on the ability of terminals to respond to multiple stimuli. Terminals with MgATP typically exhibited a capacitance increase to a second stimulus that was >70% of the amplitude of the first response and to a third stimulus with a response amplitude that was >50% of the first, whereas terminals without MgATP responded to a second stimulus with a response <35% of the first and rarely responded to a third flash. These results suggest a major role for MgATP in preparing synaptic vesicles for fusion, but indicate that cytosolic MgATP may have little role in events downstream of calcium entry, provided that $[Ca^{2+}]_i$ near release sites is elevated above ≈30 µM.

KEY WORDS: exocytosis • membrane fusion • priming • MgATP • synaptic vesicle

INTRODUCTION

Over the past decade, great advances have been made in understanding the mechanisms of neurotransmitter exocytosis. The identification of a number of highly conserved proteins that are important for membrane trafficking and fusion, combined with the development of new techniques for monitoring exocytosis have permitted models of the molecular events leading up to the calcium-dependent fusion of synaptic vesicles to be proposed. The soluble NSF attachment protein receptor (SNARE)[1] hypothesis (Söllner et al., 1993*a*) has proved an important concept for the development of these models, but there remains uncertainty about the role and relative order of some of the proposed steps. One area of investigation that has received much attention is the ATP-dependent step(s) in neurotransmitter exocytosis.

The importance of MgATP in exocytosis is well established. While early reports in permeabilized neuroen-docrine cells reported both MgATP-dependent and -independent release of catecholamines, upon closer investigation it became clear that the so-called MgATP-independent component had already been exposed to MgATP and therefore did not require cytosolic MgATP at the time of stimulation (Holz et al., 1989; Hay and Martin, 1992). This finding led to the hypothesis that MgATP is necessary for the "priming" of vesicles for fusion and that the calcium-dependent triggering step for exocytosis must occur after the ATP-dependent priming step (Holz et al., 1989; Hay and Martin, 1992). This sequence of steps, ATP-dependent priming preceding calcium-dependent fusion, is supported by the results of several studies in which the photolyzable calcium chelator DM-nitrophen was used to elevate internal calcium. In addition to having a high affinity for calcium, DM-nitrophen has a high affinity for magnesium (Kaplan and Ellis-Davies, 1988), and therefore unless particular care is taken when designing the internal solution, free cytosolic magnesium will be very low (e.g., Parsons et al., 1995). Despite greatly decreased cytosolic magnesium, flash photolysis of calcium caged with DM-nitrophen was found to produce an exocytotic response in synaptic terminals (Heidelberger et al., 1994) and neuroendocrine cells (Neher and Zucker, 1993; Thomas et al., 1993; Heinemann et al., 1994; Parsons et al., 1995). Furthermore, the final steps in the exocytotic pathway could be modeled with a simple cal-

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[1]*Abbreviations used in this paper:* Gm, membrane conductance; Gs, series conductance; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNARE, soluble NSF attachment protein receptor.

cium-binding scheme that places the calcium-dependent steps immediately before the final fusion step (Heidelberger et al., 1994; Heinemann et al., 1994). Consistent with the interpretation that the last ATP-dependent reaction occurs before calcium-triggered fusion are the results of a recent study in chromaffin cells, which combined physiological and morphological approaches to place the last ATP-dependent priming step very close to the docking of granules with the plasma membrane and well before calcium-triggered fusion (Parsons et al., 1995). Not all of the granules that were docked were found to be fusion competent, however. It seems likely that chromaffin granules must undergo additional maturational steps to achieve full fusion competence (Parsons et al., 1995; Steyer et al., 1997). A similar suggestion has been made for synaptic vesicles (Südhof, 1995). Based upon the above information, a reasonable sequence for the steps in exocytosis could be summarized as docking, followed by ATP-dependent priming and vesicle maturation, calcium entry, and fusion and release of vesicular contents (Augustine et al., 1996).

Biochemical evidence provides equally compelling support for a role of MgATP in membrane fusion. The N-ethylmaleimide-sensitive fusion protein (NSF), an ATPase identified as an essential component in studies of membrane trafficking and vesicle transport through the Golgi apparatus (Glick and Rothman, 1987; Orci et al., 1989), was subsequently proposed to be part of a generalized fusion mechanism common to both constitutive and regulated membrane fusion (Söllner et al., 1993a; Rothman, 1994). Hydrolysis of ATP by NSF catalyzes the rearrangement of the 20S SNARE complex, causing it to disassemble, and this disassembly reaction is thought to be an essential step in the exocytotic process, possibly initiating fusion (Rothman and Orci, 1992; Söllner et al., 1993b). At what point in the secretory pathway this important disassembly step occurs is currently an area of investigation. Recently, it was shown in permeabilized PC12 cells that the disassembly of the 20S complex by the hydrolysis of ATP by NSF may occur at a prefusion step rather than at fusion itself (Banerjee et al., 1996). Furthermore, two studies of vacuolar fusion in yeast have demonstrated a functional role for ATP hydrolysis by NSF before docking (Mayer et al., 1996; Nichols et al., 1997). Identifying the position of the ATP-dependent step(s) in the exocytotic sequence is further complicated by suggestions that there is likely to be more than a single ATP-dependent reaction in exocytosis. For example, an ATP-dependent priming step involving phosphorylation by myosin light chain kinase has been reported in chromaffin cells (Kumakura et al., 1994), and, in PC12 cells, Hay et al. (1995) and Martin et al. (1995) have identified a novel ATP-dependent priming reaction that requires the phosphorylation of a phospholipid. Whether either of the above phosphorylation reactions is important for synaptic vesicle fusion is still unknown. Additionally, a newly identified ATPase, Hrs-2, has been implicated in the regulation of calcium-dependent secretion (Bean et al., 1997).

In the present study, a physiological approach was taken to examine the role of MgATP in exocytosis in single synaptic terminals of retinal bipolar neurons. Unlike neuroendocrine cells, in which catecholamines are typically released from large dense-core granules, these neurons are glutamatergic (Marc et al., 1990) and contain small ($\sim$29 nm diameter), clear-core synaptic vesicles (von Gersdorff et al., 1996). Biophysical techniques with high temporal resolution were used to assess the kinetics of calcium-dependent synaptic vesicle fusion in order to determine whether or not there is a requirement for cytosolic MgATP late in the neuronal secretory pathway. Such a requirement would be missed with lower temporal resolution approaches. No clear role for cytosolic MgATP was identified in the steps of the secretory pathway that occur subsequent to the elevation of intraterminal calcium. However, cytosolic MgATP was found to be important for a process that occurs before calcium entry and may be related to the priming of synaptic vesicles for calcium-dependent fusion.

MATERIALS AND METHODS

Cell Isolation and Solutions

The dissociation procedure for the acute isolation of Mb1 bipolar neurons and their synaptic terminals has been described in detail (Heidelberger and Matthews, 1992). Briefly, dark-adapted goldfish, 8–12 cm in length, were decapitated and enucleated. The lens and vitreous were removed in cold, oxygenated Ringer solution containing (mM): 120 NaCl, 2.6 KCl, 1.0 MgCl$_2$, 0.5 CaCl$_2$, 11 glucose, and 10 HEPES, pH 7.3. Eyecups were then incubated in hyaluronidase, 1,100 U/ml, for 30 min to digest the remaining vitreous, and then rinsed. Retinae were removed and chopped into small pieces ($\approx$1 mm^2). The pieces were incubated for $\sim$30 min in solution containing (mM): 115 NaCl, 2.5 KCl, 1.0 MgCl$_2$, 0.5 CaCl$_2$, 11 glucose, 10 piperazine-N,N'-bis(2-ethane sulfonic acid) (PIPES), 2.7 cysteine, pH 7.3, and papain (Fluka, Buchs, Switzerland), $\approx$30 U/ml. After enzymatic treatment, pieces were rinsed several times and stored for up to 10 h in cold, oxygenated Ringer solution. Pieces were mechanically triturated as needed onto clean glass coverslips. Isolated bipolar somata and isolated synaptic terminals were identified on the basis of their characteristic appearance, size (terminals: 8–12 μm diameter; Ishida et al., 1980), and electrophysiological profile (Kaneko and Tachibana, 1985; Heidelberger and Matthews, 1992; von Gersdorf and Matthews, 1994).

External bathing solution for all experiments contained (mM): 115 NaCl, 2.6 KCl, 1.6 MgCl$_2$, 1.0 CaCl$_2$, 10 HEPES, and 11 glucose, pH 7.3, 260 mosM. The composition of the internal recording solutions is shown in Table I. DM-nitrophen (Kaplan and Ellis-Davies, 1988) was chosen over NP-EGTA (Ellis-Davies and Kaplan, 1994) as the photolyzable Ca^{2+} chelator because NP-

EGTA could not be readily photolyzed with the flash systems that were used. The internal solutions were designed so that DM-nitrophen was maximally loaded with divalent cations, >80% of which are represented by Ca^{2+}, to minimize the possibility of a calcium transient caused by the rebinding of photoreleased Ca^{2+} to unloaded and unphotolyzed DM-nitrophen. Simulations of the calcium time course after a flash were performed and failed to predict a calcium spike with these solutions. An aliquot of every solution was removed before the addition of furaptra and spiked with 200 μM fura-2 for an in vitro droplet measurement of free $[Ca^{2+}]$. The free Ca^{2+} was carefully adjusted to <2 μM in Solutions C, D, and E by adding small volumes of DM-nitrophen or $CaCl_2$. Because of the complex solution chemistry of Solutions A, B, and F, those with a measured free $Ca^{2+} > 2$ μM were discarded, as were all ATP-containing solutions that failed to support the recovery of I_{Ca} after inactivation. The pH and osmolarity of the final internal solutions were adjusted to 7.3 and 265 mosM, respectively. Cs^+ salts were prepared from CsOH purchased from Aldrich Chemical Co. (Steinheim, Germany). Furaptra and fura were obtained from Molecular Probes, Inc. (Eugene, OR). DM-nitrophen was obtained as a gift from the laboratory of Dr. Jack Kaplan (Oregon Health Sciences University, Portland, OR) and purchased from Calbiochem Corp. (Bad Soden, Germany). ATP-γ-S and GTP were obtained from Boehringer Mannheim (Mannheim, Germany). All other reagents were obtained from Sigma Chemical Co. (Deisenhofen, Germany). Internal solutions were protected from light and kept on ice when not stored at −70°C.

Electrical Measurements

Conventional whole-cell recordings were performed on isolated synaptic terminals using sylgard-coated pipettes with resistances

of 6–10 MΩ. Vhold was −60 mV. All experiments were performed at room temperature (21–24°C). Terminals with leak current >40 pA were excluded from the data pool.

A computer-controlled EPC-9 patch-clamp amplifier (HEKA Electronik, Lambrecht, Germany) was used to make electrical recordings. High resolution capacitance measurements were performed with either the EPC-9 software or with IGOR (Wavemetrics, Inc., Lake Oswego, OR) and Pulse Control XOPS (Jack Herrington and Richard Bookman, University of Miami, Miami, FL). A sinusoidal stimulus, ≤32 mV peak-to-peak, was applied about the DC holding potential. Sine wave frequencies were from 1,000 to 3,200 Hz. The resulting current was processed using the Lindau-Neher technique (Lindau and Neher, 1988; Gillis, 1995) to give estimates of the equivalent circuit parameters (Cm, Gs, Gm). The reversal potential of the measured DC current was assumed to be 0. One capacitance data point was generated and collected per sine wave cycle in high resolution mode. For time-resolved measurements, the capacitance data points collected during a 100-ms sweep were averaged to yield one capacitance data point per sweep. Alternatively, in a few experiments, time-resolved capacitance measurements were made using the automatic capacitance compensation of the EPC-9 amplifier (Chow et al., 1992; Heidelberger et al., 1994).

Flash Photolysis of Caged Ca^{2+} and $[Ca^{2+}]_i$ Measurements

For measurement of $[Ca^{2+}]_i$, alternating excitation at 345 and 390 nm was typically provided by a computer-controlled monochrometer-based system (T.I.L.L. Photonics, Gräfeling, Germany) as described by Messler et al. (1996). For high resolution measurements, the exposure duration at 345 and 390 nm was 20 and 50 ms, respectively. Each cycle of alternating excitation was

TABLE I
Composition of the Internal Recording Solutions

Solution	A	B	C	D	E	F
Na$_4$-DM-nitrophen	10	10	10	10	10	10
CaCl$_2$	8	8	10	10	10	8
MgCl$_2$	4	4	0	0	0	4
LiCl	0	0	0	0	40	0
Na$_2$-ATP	10	0	0	0	0	10
Li$_4$-ATP-γ-S	0	10	2	0	0	0
K$_2$-DTPA	0	0	5	5	5	0
GTP	0.5	0.5	0.5	0	0.5	0
Furaptra	1	1	1	1	1	1
Cs-glutamate	0	0	0	62	0	55
Cs-gluconate	19	21	18	0	25	0
Cs-HEPES	56	62	54	31	75	28
TEA-Cl	4.9	5.5	4.8	6.24	6.6	5.54
Free $[Ca^{2+}]$	≈250 nM	≈250 nM	≈0	≈0	≈0	≈250 nM
[MgATP]	≈2 mM	≈2 mM	≈0	≈0	≈0	≈2 mM

The ATP-containing internal solutions were designed to meet the following minimum criteria: free $[Ca^{2+}] < 2$ μM, [MgATP] > 1 mM, and DM-nitrophen fully loaded with divalent cations, >80% of which are represented by Ca^{2+}. Because ATP is a significant Ca^{2+} buffer ($K_d \approx 110$ μM, Smith and Martell, 1989), no additional low affinity Ca^{2+} buffer was added to ATP-containing solutions. For solutions that contained ≤2 mM added nucleotide, DPTA (1,3-diaminopropane-2-of-*N,N'*-tetraacetic acid, $K_d \approx 81$ μM; Neher and Zucker, 1993) was added as the low affinity Ca^{2+}-buffer. Values for free $[Ca^{2+}]$ and [MgATP] were calculated with an algorithm adapted from Trube et al. (1979). It is assumed that ATP-γ-S has a similar affinity for Ca^{2+} as ATP. Solution E was developed to test for possible effects of lithium, which is contained in the ATP-γ-S solutions, on exocytosis. Calculations show that for Solution A, ~98% of the furaptra is free, despite the presence of Mg^{2+}. After 50% photolysis of DM-nitrophen, >98% of the divalent cations bound to furaptra are calculated to be Ca^{2+} ions. Therefore furaptra should give as faithful an estimate of free $[Ca^{2+}]$ with the Mg^{2+}-containing internal solutions as it does for solutions without Mg^{2+}. All concentrations are listed in millimolar.

separated from the next by 30 ms of darkness, and one calcium point was generated for each value at 390 nm. For time-resolved measurements, exposure duration during each 100-ms sweep was 20 and 25 ms at 345 and 390 nm, respectively, and one calcium point was generated per sweep. In some experiments, alternating excitation was provided by a two-flash-lamp system (T.I.L.L. Photonics). With both systems, the intensity of the excitation was reduced far below the level at which a calcium increase due to photolysis of DM-nitrophen by this excitation light could be observed in in vitro and in vivo assays. The field stop of the microscope was set so that an ∼70-μm area in the specimen plane was illuminated, and the optical coupling was optimized so that the illumination was homogeneous in intensity. Emitted fluorescence was collected from an ∼20-μm-diameter spot in the object plane through a 470-nm long-pass and a 540-nm short-pass filter and, for the monochrometer-based system, detected by a photomultiplier tube (R928; Hamamatsu Phototonics, Hamamatsu City, Japan). This fluorescence signal was sampled by the EPC-9 and acquired using the fura extension of the Pulse software (HEKA Electronik, Lambrecht, Germany). For the two-flash-lamp system, both excitation and emitted light were detected by photodiodes, simultaneously integrated and digitized as described by Heinemann et al. (1994). Flashes of UV light, derived from a Xenon arc flash lamp (Gert Rapp Optoelektronik, Hamburg, Germany) were coupled through the epifluorescence port of either a Zeiss Axiovert 100 or a Zeiss IM 35 (Carl Zeiss, Inc., Oberkochem, Germany) microscope by means of a sapphire window (Steeg and Reuter, Geissen, Germany). This device combined the light from the flash lamp, with 85% transmission efficiency, with the excitation light for measurement of intraterminal calcium, which was reflected with 15% efficiency. $[Ca^{2+}]_i$ was ratiometrically calculated by the method of Grynkiewicz et al. (1985). Calibration constants were obtained as described by Heinemann et al. (1994) and take into account the bleaching of furaptra and DM-nitrophen after a UV flash. For the monochrometer-based system, Rmin and Rmax were found to be 0.105 and 3.972, respectively, and were not significantly altered by a UV flash. Keff ranged from a preflash value of 2.204 to 5.014 mM with a maximum intensity

UV flash. For the two-flash-lamp system, Rmin was 0.329 and Rmax was 6.678, and Keff ranged from 1.709 to 2.119 mM, depending upon the flash intensity.

RESULTS

Flash Photolysis of Caged Calcium in Combination with Capacitance Measurements

Flash photolysis of caged calcium has become a useful tool with which to study the kinetics of exocytosis (Neher and Zucker, 1993; Thomas et al., 1993; Heinemann et al., 1994; Heidelberger et al., 1994). Recently, however, a concern has been raised as to whether or not the change in capacitance evoked by photorelease of calcium is due to the addition of membrane that is related to synaptic vesicle fusion and neurotransmitter exocytosis (Oberhauser et al., 1995, 1996). Two sets of experiments were designed to address this issue in synaptic terminals of Mb1 retinal bipolar neurons. As a first step towards determining whether the membrane that is added in response to flash photolysis of caged calcium is from a pool of membrane that is synapse specific, the capacitance responses of synaptic terminals of bipolar neurons were compared with the capacitance responses of somata of bipolar neurons. Elevation of intraterminal calcium was elicited by the flash photolysis of calcium-loaded DM-nitrophen using maximum intensity UV flashes. Such flashes are expected to raise calcium >>100 μM and achieve maximal rates of synaptic vesicle fusion (Heidelberger et al., 1994). Fig. 1 demonstrates the response of a single synaptic terminal
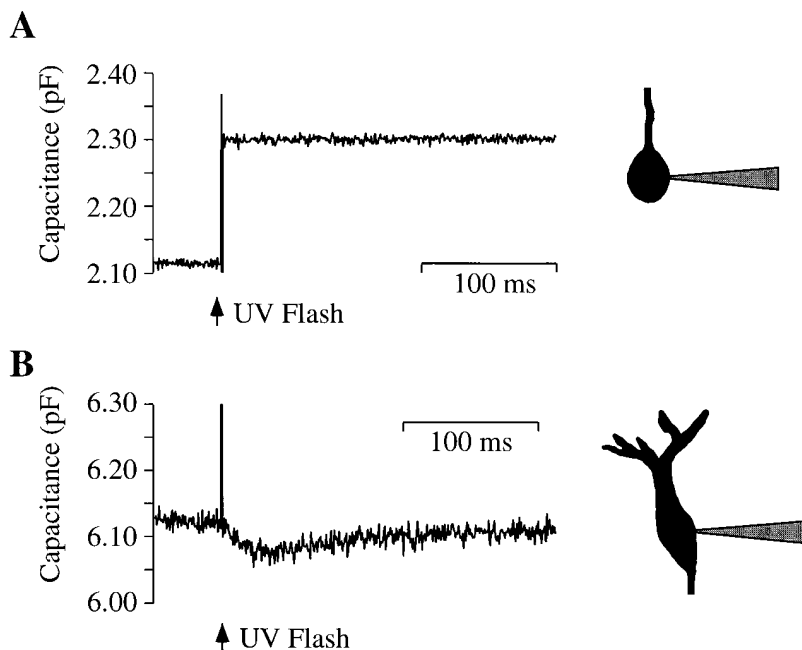

FIGURE 1. Synaptic terminals, but not somata, respond to elevation of $[Ca^{2+}]_i$ with an increase in membrane capacitance. (A) The high resolution capacitance record of an isolated synaptic terminal from a bipolar neuron is shown. At the arrow, a 375-V UV flash was given to photolyze calcium-loaded DM-nitrophen. Immediately after the electrical artifact (i.e., spike) in the capacitance record caused by the ignition of the flash lamp, the membrane capacitance increased rapidly by 186 fF. The rate of membrane addition is consistent with the elevation of intraterminal calcium to a concentration >>100 μM (Heidelberger et al., 1994). (B) The capacitance record from an isolated bipolar neuron soma that lost its axon and terminal during the dissociation process is shown. After an identical 375-V UV flash and the resultant electrical artifact (i.e., spike), the capacitance of the soma slowly decreased by 45 fF, and then slowly returned to baseline. There is no indication that membrane was added. Soma is from the same preparation as the terminal in A. For both A and B, the internal recording solution used was Solution F.

and a bipolar neuron soma to the rapid elevation of intracellular calcium. Consistent with previously published findings (Heidelberger et al., 1994), the synaptic terminal exhibited a rapid increase in membrane capacitance in response to flash photolysis of caged calcium (Fig. 1 *A*). In contrast, the soma, whose axon and synaptic terminal had been lost during the dissociation process, did not exhibit an increase in capacitance in response to the elevation of intracellular calcium (Fig. 1 *B*). Like several of the somata examined, this soma exhibited a slow decrease in membrane capacitance after the flash that was not examined further. Four pair-wise comparisons across two preparations were performed. In all instances, the synaptic terminal responded with a rapid increase in membrane capacitance. However, somata never showed a capacitance increase. The fact that synaptic terminals of bipolar neurons, and not somata, respond to an elevation in intracellular calcium with the addition of membrane indicates that the pool of membrane that undergoes calcium-dependent fusion is specifically localized to the synaptic terminal.

Next, "cross-depletion" experiments were performed to ascertain whether the synaptic-specific pool of membrane that is added in response to flash photolysis of caged calcium comes from the same pool of membrane that fuses in response to a physiological stimulus. To this end, capacitance responses to UV flashes and membrane depolarizations were compared within single synaptic terminals. In bipolar neuron synaptic terminals, the presynaptic calcium channel is an L-type calcium channel (Heidelberger et al., 1992; Tachibana et al., 1993), and similar to what has been described for other L-type calcium channels (e.g., Chad and Eckert, 1986; Armstrong and Eckert, 1987; Armstrong et al., 1991), MgATP must be present in the internal solution for the calcium current to recover after inactivation (Heidelberger and Matthews, unpublished observations). Therefore, the DM-nitrophen-containing internal was designed to contain a concentration of MgATP >1 mM. Because DM-nitrophen binds magnesium with a fairly high affinity ($K_d \approx 2.5$ μM; Kaplan and Ellis-Davies, 1988), ATP was added in a concentration equal to that of DM-nitrophen (Table I, Solution F) to establish a competition for magnesium between ATP and DM-nitrophen. With this solution, MgATP was calculated to be ~2 mM and free calcium to be ~250 nM at steady state. The free calcium was verified with fura-2 in an in vitro assay, and the ability of the solution to support recovery of the calcium current from inactivation was confirmed (data not shown). To perform the cross-depletion, a 2-s depolarization from −60 to 0 mV was given to deplete the terminal of all the vesicles that are available for release. Then, after a variable length of time, Δt_1, a near-maximal UV flash was given. This was

followed by a second variable period of time, Δt_2, and an additional stimulus, which could be either a pulse or a flash (Fig. 2 *A*). The cross-depletion was not performed in the opposite manner (flash followed immediately by depolarization) because the time required for the calcium current to recover from inactivation after a flash was found to be greater than the time required to refill the release-ready pool after depletion by the flash (see also von Gersdorff and Matthews, 1996, 1997). Fig. 2 *B* shows a typical experiment that resulted from following the protocol depicted in Fig. 2 *A*. In response to the first depolarizing voltage pulse, the membrane capacitance increased by 487 fF. A flash given 5 s later evoked an additional capacitance increase of only 67 fF. In contrast, when an identical UV flash was given ~126 s later, the increase in membrane capacitance evoked by the flash was 179 fF. The fourth stimulus, a depolarizing pulse, was given later still and evoked a capacitance increase of 91 fF. The fact that the calcium current was sufficiently large to evoke a capacitance response in a terminal that was subjected twice to the global elevation of intraterminal calcium to concentrations >>100 μM and once to the local elevation of in-
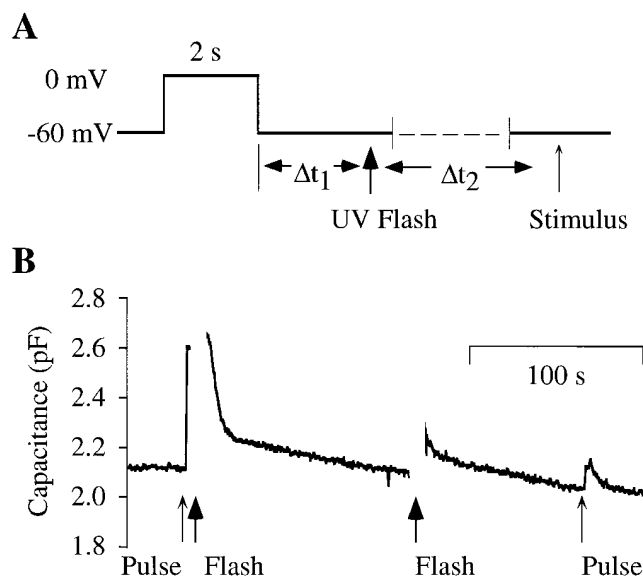

FIGURE 2. Cross-depletion studies in a single synaptic terminal. (*A*) A cross-depletion paradigm was implemented to determine whether the elevation of intraterminal calcium by the opening of voltage-gated calcium channels and flash photolysis of caged calcium evokes fusion of membrane from the same pool. The protocol used to perform the cross-depletion is illustrated. (*B*) A time-resolved capacitance record of a cross-depletion experiment in a single synaptic terminal. 2-s depolarizations from −60 to 0 mV were alternated with 300-V UV flashes. The pipette solution was Solution F. Gaps in the capacitance record indicate the time period during which high resolution capacitance measurements were made (not shown).
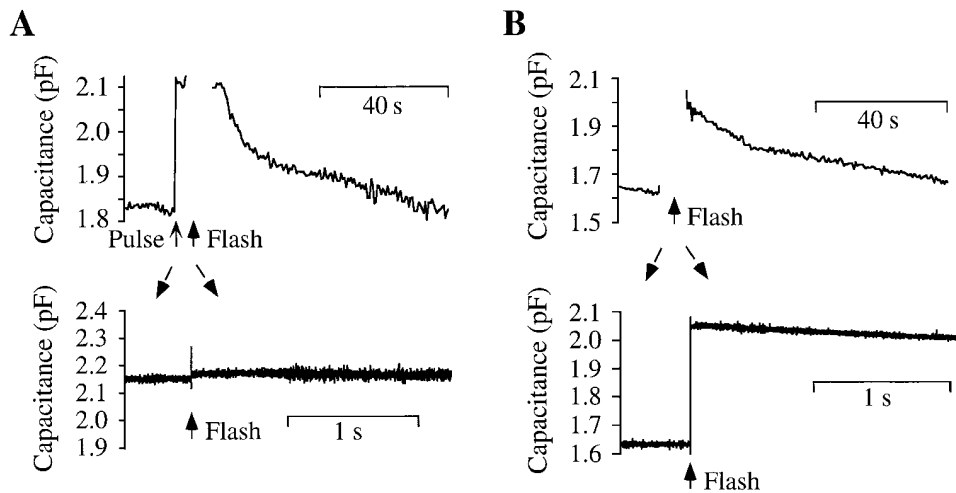
FIGURE 3. Comparison of high resolution capacitance records elicited by the cross-depletion paradigm. (A) Top is the time-resolved capacitance record of a terminal given a 2-s depolarization from −60 to 0 mV, followed within 5 s by a 300-V UV flash. In response to the pulse, the membrane capacitance increased by 289 fF. The gap in the top trace indicates the time period during which high resolution capacitance measurements were made. The resulting high resolution measurement is shown on the bottom. The electrical artifact (i.e., spike) and the arrow below the trace indicate the timing of the flash. Only 16 fF was added by this flash, which was preceded by a depolarization. (B) Time-resolved and high resolution capacitance records from a terminal that was stimulated with a 300-V UV flash that was not preceded by a depolarization. After the flash, a 416-fF increase in membrane capacitance can be observed. The gap on the top trace indicates the time period during which high resolution capacitance measurements were made. The resulting high resolution measurement is shown on the bottom. The electrical artifact (i.e., spike) and the arrow below the trace indicates the timing of the flash. The terminal is from the same preparation as the terminal in A.

traterminal calcium by a long, strong depolarization confirms that sufficient MgATP was present in the internal solution to maintain the calcium current. Fig. 3 shows examples of other cross-depletion experiments at high temporal resolution. In the first example, when the flash was preceded by a pulse, the pulse evoked a capacitance increase of 289 fF and the flash evoked a capacitance increase of only 16 fF (Fig. 3 A). In contrast, a flash given alone evoked a capacitance increase of 416 fF (Fig. 3 B, different terminal). In seven such cross-depletion experiments, the capacitance increase triggered by a flash alone averaged 161 ± 42 fF (mean ± SEM; Fig. 4 A). The 2-s depolarization alone evoked an average increase of 142 ± 39 fF. When the flash was immediately preceded by the 2-s pulse, ($\Delta t < 5$ s), the capacitance increase evoked by the flash was only 28 ± 10 fF ($n = 6$). This small amount of membrane added within the 5-s interval may be attributed to refilling of the release-ready pool, which proceeds with a time constant of 8 s (von Gersdorff and Matthews, 1997). To express the results another way, the amplitude of the capacitance response to a flash, separated by at least 60 s from a depolarizing pulse, was normalized by the amplitude of the response to the pulse. This flash/pulse ratio was 1.01 ± 0.17 (mean ± SEM; Fig. 4 B), indicating that the size of the pool of membrane added with either mode of stimulation is identical. However, when a flash followed a pulse by <5 s, this ratio fell to 0.10 ± 0.02 (Fig. 4 B). These results suggest that flash photolysis of caged calcium taps the same pool of membrane as calcium influx through voltage-gated calcium channels. The latter stimulus is known to trigger glutamate release from these terminals (Tachibana et al., 1993).

Time-resolved and High Resolution Capacitance Measurements with and without MgATP

Having provided evidence to suggest that in the bipolar neuron synaptic terminal, flash photolysis of caged calcium can be combined with capacitance measurements to study synaptic vesicle fusion, the exocytotic response was compared in the presence of internal MgATP or MgATP-γ-S. Fig. 5 shows an experiment from a terminal that was internally dialyzed with internal solution containing MgATP. The capacitance record at low temporal resolution is shown in Fig. 5 A. Each UV flash resulted in the liberation of calcium from DM-nitrophen and an increase in membrane capacitance. The largest capacitance response is shown at higher temporal resolution in Fig. 5 B along with the corresponding membrane conductance (Gm) and series conductance (Gs) traces. It can be seen that little change occurs in Gm and Gs after the flash, indicating that the observed increase in membrane capacitance is due to the addition of membrane rather than another change in the equivalent circuit. The high resolution calcium trace corresponding to the high resolution capacitance record is shown in Fig. 5 C. Before the flash, the resting calcium concentration was below the limit of detectability for the low affinity calcium indicator dye furaptra. After the flash, the intraterminal calcium increased. The average postflash calcium during the sweep shown was 33 μM, as is indicated by the dotted line. Identical experiments were performed in terminals dialyzed with internal solution that contained MgATP-γ-S (Fig. 6). This terminal was given two identical UV flashes (Fig. 6 A). The first flash elevated the intraterminal calcium to 30
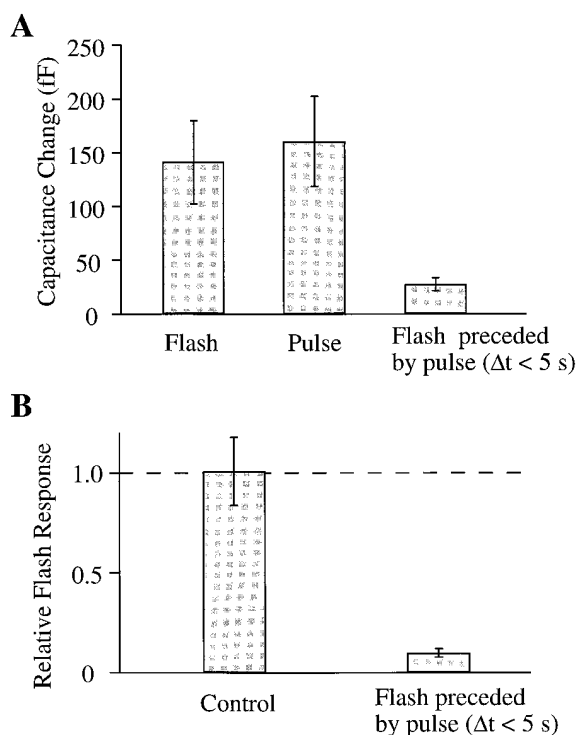
FIGURE 4. Flash photolysis of caged calcium triggers fusion of synaptic vesicles from the same pool of vesicles as activation of I_{Ca}. (*A*) The mean amplitudes of the capacitance increase in response to a 300-V UV flash, a 2-s depolarization from -60 to 0 mV, and a 300-V UV flash that was given no more than 5 s after a 2-s depolarization from -60 to 0 mV are represented. Error bars indicate $\pm$ 1 SEM. (*B*) For each terminal, the amplitude of the capacitance increase evoked by a UV flash was normalized by the amplitude of the capacitance response evoked by a 2-s depolarization from -60 to 0 mV. The first bar indicates the mean flash/pulse ratio ($n = 7$). The second bar is the normalized amplitude of the capacitance increase in response to a UV flash in a terminal that was stimulated <5 s before with a 2-s depolarization ($n = 6$). Error bars represent $\pm$ 1 SEM.

μM (Fig. 6 *C*), and the second flash increased the calcium to 26 μM (data not shown). However, an increase in membrane capacitance was observed only in response to the first flash (Fig. 6 *A*). This capacitance response is shown at high resolution in Fig. 6 *B*, along with the relevant Gm and Gs traces. As in the previous example, there were no changes in Gm or Gs that corresponded to the change observed in the capacitance record, indicating that the observed increase in membrane capacitance is due to the addition of membrane.

The Amplitude of the Flash Response Is Not Graded with Internal Calcium

To determine whether the amplitude of the capacitance response is dependent upon the postflash intraterminal calcium concentration, the level to which the internal calcium was raised was controlled by adjusting
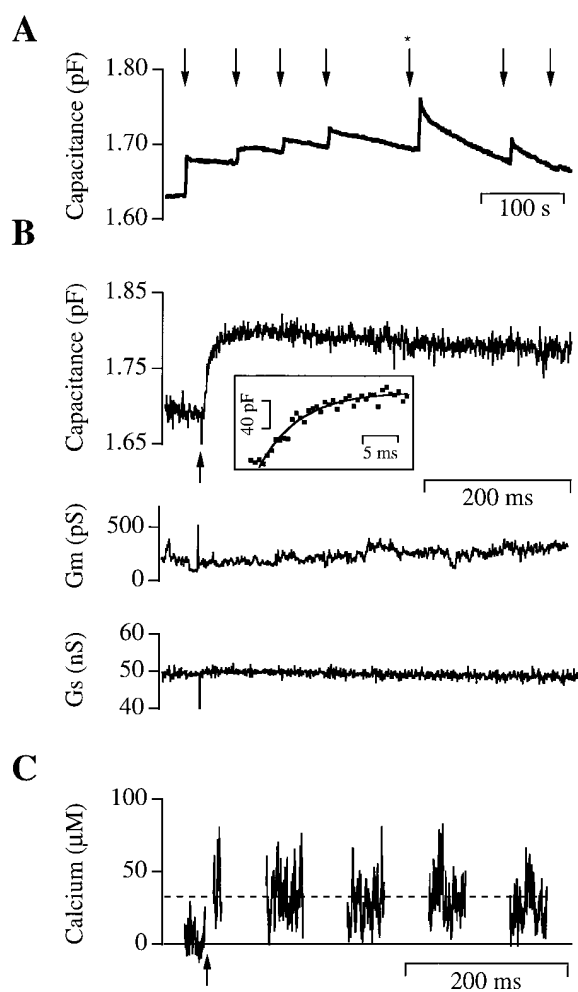

FIGURE 5. Results from a single synaptic terminal with MgATP. (*A*) Time-resolved capacitance record from a synaptic terminal loaded with internal Solution A via the whole-terminal recording electrode. At each arrow, a UV flash was given to elevate the intraterminal calcium concentration above the threshold for secretion. The first flash was given $\sim$2 min after break-in. The terminal responded to each elevation with an increase in membrane capacitance. (*B*) Top shows the high resolution capacitance record of the flash response indicated by the asterisk in *A*. Note the time scale. Timing of the UV flash is indicated by the arrow. After the flash, the capacitance increased by 97 fF. The inset shows the rising the phase of the capacitance increase more clearly. The curve fitted to the capacitance response was drawn according to C (in pF) $= 1.79$–$0.186(\exp[-at])$, where t is the time after the flash and $a = 168$ s^{-1}. The bottom shows the membrane conductance and series conductance records at the same time scale as the high resolution capacitance record. There is no change in either the membrane conductance or series resistance after the UV flash that correlates with the observed increase in membrane capacitance. (*C*) The intraterminal calcium concentration was ratiometrically calculated from the alternating excitation of furaptra at 345 and 390 nm. The elevation of calcium after the flash gave rise to the capacitance response shown in *B*. The dotted line indicates the average value of the postflash intraterminal calcium concentration (33 μM). Gaps in the calcium trace indicate intervals in which furaptra was not excited at 390 nm and includes the excitation interval at 345 nm and the dark period between excitations. Additionally, a 10-ms period immediately after the flash, which allows time for the photomultiplier to recover from the flash, has been blanked. Timing of the flash is indicated by the arrow.
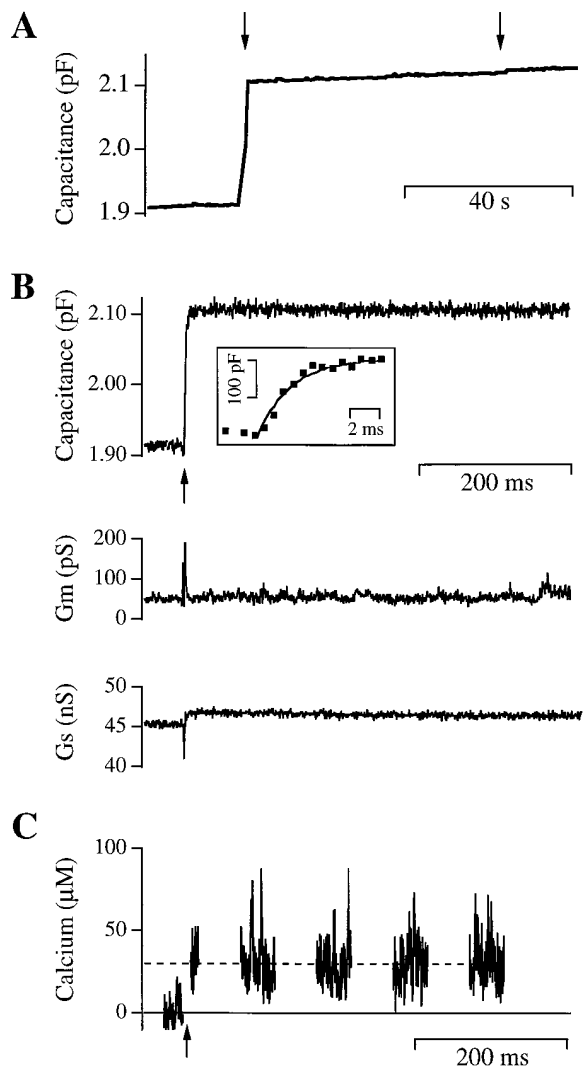
FIGURE 6. Results from a single synaptic terminal dialyzed with MgATP-γ-S. (*A*) Time-resolved capacitance record of a single synaptic terminal that was loaded via the patch pipette with Solution B. At each arrow, a UV flash was given to elevate the intraterminal calcium concentration above threshold. The first flash was given ~1 min after break-in. Only the first flash evoked an increase in membrane capacitance. Terminal is from the same preparation as that in Fig. 5. (*B*) Top shows the high resolution capacitance record of the response in *A*. Note the time scale. Timing of the UV flash is indicated by the arrow. After the flash, the capacitance increased by 193 fF. The inset shows the rising phase of the capacitance response at an expanded time scale. The curve fitted to the capacitance response was drawn according to *C* (in pF) = 2.11–0.587(exp[−*at*]), where *t* is the time after the flash and *a* = 482 s^{-1}. The bottom shows the corresponding membrane and series conductance records. Neither exhibits a significant change after the flash that correlates with the change in capacitance. (*C*) The intraterminal calcium concentration was ratiometrically calculated from the alternating excitation of furaptra at 345 and 390 nm. The elevation of calcium after the flash gave rise to the capacitance response shown in *B*. The dotted line indicates the average value of the postflash intraterminal calcium concentration (30 μM). Gaps in the calcium trace indicate intervals in which furaptra was not excited at 390 nm and includes the excitation interval at 345 nm and the dark period between excitations. Additionally, a 10-ms period

immediately following the flash, which allows time for the photomultiplier to recover from the flash, has been blanked. Timing of the flash is indicated by the arrow.

the voltage setting of the flash lamp and by placing neutral density filters in the UV-flash light path. Internal calcium was determined with the low affinity calcium-indicator dye furaptra. The amplitude of each capacitance response was measured and plotted against the ratiometrically determined postflash internal calcium concentration. In terminals given multiple flashes, only the first capacitance was included in the analysis in order to avoid complications of globally elevated internal calcium upon subsequent responses. Above the calcium threshold for exocytosis, no correlation was found between the amplitude of the first capacitance response and the postflash intraterminal calcium concentration (Fig. 7). Rather, there was a random distribution of amplitudes. A lack of correlation between the size of the exocytotic burst and postflash calcium has also been reported for pituitary melanotrophs (Thomas et al., 1993) and bovine adrenal chromaffin cells (Gillis et al., 1996). This is different than what has been reported for exocytosis in synaptic terminals that is evoked by membrane depolarizations of increasing length, where the size of the exocytotic response increases until a plateau is reached (e.g., von Gersdorff and Matthews, 1994). This apparent discrepancy can be explained by considering the manner in which calcium is elevated. A very brief membrane depolarization may only allow in enough calcium to trigger the release of a small fraction of the available vesicles. Because the change in membrane capacitance is measured after the cessation of the depolarization, as the length of the depolarization increases, not only do more calcium ions enter, but these ions have more time in which to diffuse away from the site of entry and trigger the fusion of more distant vesicles in the release-ready pool. Thus, the amplitude of the capacitance response will grow with increasing duration of membrane depolarization until the release-ready pool is depleted. In contrast, when calcium is globally elevated above threshold by a UV flash, all of the vesicles available for release simultaneously see the rise in internal calcium and are stimulated to fuse. With this interpretation, the amplitude of the capacitance response evoked by flash photolysis of caged calcium provides information about the size of the release-ready pool of synaptic vesicles at the precise instant in time in which calcium is elevated. This interpretation is consistent with available data and is the commonly accepted interpretation (e.g., Gillis et al., 1996).
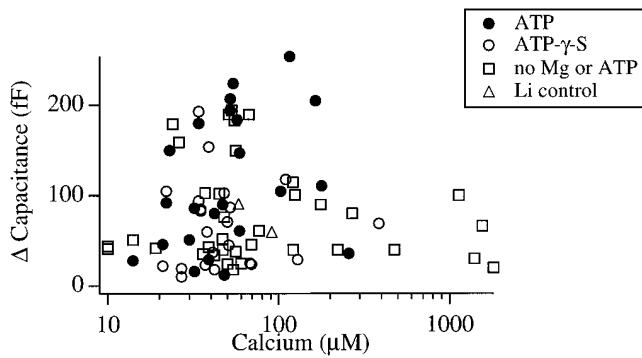
FIGURE 7. Above threshold there is no relationship between the magnitude of the capacitance increase and postflash $[Ca^{2+}]_i$. For each terminal, the amplitude of the capacitance response evoked by the first UV flash was plotted against the postflash intraterminal calcium concentration. (●) The first flash response of terminals dialyzed with Solution A. (○) The first flash response of terminals dialyzed with ATP-γ-S (solution B or C). (□) The first flash response of terminals dialyzed with Solution D, which contains neither Mg^{2+}, nor added nucleotide. (△) Data from terminals loaded with Solution E, which was designed to control for the possible effects of Li^+, which is contained in the ATP-γ-S solutions, on exocytosis.

The Amplitude of the First-Flash Response with and without MgATP

The average amplitude of the first capacitance response evoked by flash photolysis of caged calcium was compared across experimental conditions. The average increase in capacitance in terminals with MgATP was found to be 121 ± 15 fF (mean ± SEM; $n = 24$), while those with ATP-γ-S showed an increase of 72 ± 10 fF (mean ± SEM, $n = 22$), and those without added Mg^{2+} or nucleotide showed an average increase of 80 ± 10 fF (mean ± SEM, $n = 34$). The former number corresponds well to reported size of the release-ready pool and the number of vesicles that are tethered to synaptic ribbons (von Gersdorff and Matthews, 1994; von Gersdorff et al., 1996). The latter values are similar to the previously published amplitude of the capacitance response in flash experiments in which the terminals were not dialyzed with magnesium or ATP (Heidelberger et al., 1994). A comparable difference between the response amplitudes in the presence or absence of ATP has been reported in flash experiments in pituitary melanotrophs (Thomas et al., 1993). This difference in the average amplitude of the capacitance response may reflect a requirement for MgATP in priming of vesicles or in replacing vesicles that have previously fused. In flash-photolysis experiments, vesicle fusion is sometimes triggered upon achieving the whole-cell recording configuration, when cytosolic Mg^{2+} displaces Ca^{2+} from DM-nitrophen, leading to a brief elevation in internal calcium termed the "loading transient" (Neher and Zucker, 1993; Parsons et al., 1996). The

loading transient, combined with an impaired ability to refill the release-ready pool, might account for a smaller flash response in terminals with ATP-γ-S relative to ATP. Additionally, some amount of vesicle "depriming" may occur in the absence of cytosolic MgATP or magnesium (Holz et al., 1989; Parsons et al., 1995) and contribute to a reduction in the size of the release-ready pool. Under experimental conditions that do not produce a loading transient or chelate cytosolic magnesium, the amplitude of the first capacitance response is virtually identical in terminals dialyzed with MgATP or MgATP-γ-S (Heidelberger and Matthews, 1997).

Exocytosis after Depletion of the Release-ready Pool Requires MgATP

One of the most striking differences between the responses of terminals dialyzed with internal solution containing MgATP compared with those dialyzed with either solution containing ATP-γ-S or no added Mg^{2+} or nucleotide is in the number of times a capacitance increase could be evoked. This is demonstrated by the pair of terminals shown in Figs. 5 and 6. The terminal with MgATP responded to each of seven flashes with an increase in membrane capacitance (Fig. 5 A), whereas the terminal with MgATP-γ-S responded only to the very first flash. The observation that terminals with MgATP respond better to subsequent stimuli than terminals with ATP-γ-S was a consistent one and is summarized in Fig. 8. Intraterminal calcium was elevated by UV flashes, and to allow time for refilling of the release-ready pool between stimulations, flashes were separated by at least ∼50 s, which is more than six times the estimated time constant for pool refilling in these terminals under standard experimental conditions (von Gerdorff and Matthews, 1997). While a decrement is observed under all conditions with increasing flash number, this decrement is most pronounced in terminals whose internal pipette solution lacked MgATP. For example, a clear difference between the amplitude of the second flash response in terminals with MgATP (74 ± 11 fF; mean ± SEM, $n = 16$) and ATP-γ-S (29 ± 12 fF; $n = 16$), or no added Mg^{2+} or nucleotide (15 ± 15 fF; $n = 20$) can be observed (Fig. 8). Furthermore, while the third flash response in terminals with MgATP resulted in an average capacitance increase of 54 ± 12 fF ($n = 11$), which is ∼53% of the first-flash response, the terminals with ATP-γ-S that were given a third flash responded with an increase in capacitance of only 7 ± 5 fF ($n = 8$), which is only ∼8% of the amplitude of the first-flash response. These data suggest that MgATP is required for a terminal to be able to respond to a stimulus once the release-ready pool has been depleted. These experiments do not rule out the possibility that additional factors, which may be lost in an activity-dependent manner or with time due to intraterminal
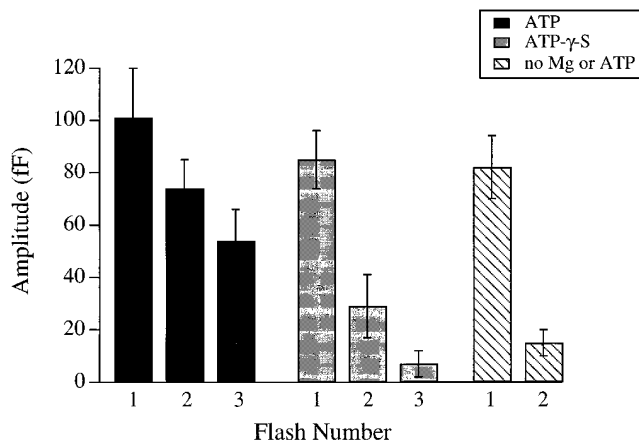
FIGURE 8. Terminals with MgATP maintain the ability to respond to stimulation better than those without. The average amplitude of the capacitance response to the first, second, and third UV flashes, given at a super-threshold intensity, is compared across three groups of synaptic terminals. Flashes were separated by a minimum of ~50 s, and typically >60 s, to allow refilling of the release-ready pool of synaptic vesicles to occur between stimulations. (*solid bars*) Data from 16 terminals dialyzed with an internal solution containing MgATP (i.e., Solution A). Only 12/16 terminals in this group were given a third flash. In the second group (*gray bars*), 16 terminals were dialyzed with an ATP-γ-S-containing solution (i.e., Solution B or C). Only 8/16 of these were given a third flash. In the final group (*hatched bars*), 21 terminals were dialyzed with Solution D, which contained neither Mg^{2+} nor nucleotide. No terminals in this group were given a third flash. Error bars indicate ±1 SEM.

dialysis, are also important for the maintenance of the capacitance response.

MgATP and the Calcium Dependence of Release

In addition to playing an important role in an earlier part of the secretory pathway related to priming or pool refilling as suggested above, cytosolic MgATP may be important in the late stages of exocytosis. To investigate this possibility, the calcium dependence of the rate of exocytosis was compared in terminals dialyzed with internal solution containing either MgATP or ATP-γ-S. As an index of the rate of secretion, an exponential function was fitted to the rising phase of each high resolution capacitance response (e.g., Figs. 5 *B* and 6 *B, insets*). Typically, the rising phase of the capacitance increase was smooth and could be fitted with a single exponential, suggesting that a homogeneous population of vesicles had fused. The rate constant ($1/\tau$) of the exponential fit was plotted against the postflash internal calcium (Fig. 9, *A* and *B*). For terminals dialyzed with internal solution containing MgATP, both the first and subsequent flash responses were included in the analysis and are denoted by different symbols (Fig. 9 *A*). No clear difference was noted in the calcium

dependence of the rate of membrane addition between the first and subsequent flashes. For terminals with ATP-γ-S, only the first-flash response was included in the analysis (Fig. 9 *B*). The approximate threshold for release can be gleaned from observing the calcium concentrations that failed to drive release compared with those that succeeded. The small symbols at the bottom of each graph, set to an arbitrary rate constant of 1 s^{-1}, indicate the maximum calcium concentration that failed to evoke a capacitance response in terminals that later responded to a larger elevation in intraterminal calcium (Fig. 9). While most maximum first-flash failures occurred in the 10–20-μM range, a few also occurred in the 30–40-μM range. In general, terminals responded with an increase in membrane capacitance when the intraterminal calcium concentration was elevated to concentrations of at least 20 μM (Fig. 9), regardless of the internal solution used. These results are consistent with previous reports examining the calcium threshold for exocytosis in these terminals (von Gersdorff and Matthews, 1994; Heidelberger et al., 1994) and suggest that the calcium threshold for release is not influenced by cytosolic MgATP in synaptic terminals.

Responses from all synaptic terminals, regardless of internal solution or flash number, exhibited a steep dependence upon the intraterminal calcium (Fig. 9). Our published model of the final steps of secretion in these terminals (Heidelberger et al., 1994), shown as a solid line (Fig. 9, *A* and *B*) fitted the data from both sets of experiments quite well, consistent with the hypothesis that there is at least a fourth order dependence of exocytosis on the intraterminal calcium concentration and that there may be cooperativity between the binding sites. Furthermore, the half-maximal rate for both experimental conditions was achieved at a calcium concentration between 100–200 μM, consistent with neuronal exocytosis being a low affinity process (Llinas et al., 1992; Heidelberger et al., 1994). The maximum rate of fusion also exhibited no dependence upon the presence or absence of MgATP or ATP-γ-S in the internal solution. Saturation of the rate constant at 2,000–3,000 s^{-1} occurred at calcium concentrations >~200 μM with either MgATP or ATP-γ-S. These data therefore suggest that, within the limits of resolution of the techniques used, the presence or absence of MgATP in the internal solution does not alter the fundamental relationship between calcium and the rate of exocytosis in synaptic terminals.

To more directly compare the calcium dependence of the rate of release in terminals dialyzed with MgATP or ATP-γ-S, data from the first-flash response were binned according to the postflash intraterminal calcium concentration, and the rate constants within each calcium range were averaged and plotted as a function of the binned calcium (Fig. 10). Data from our previ-
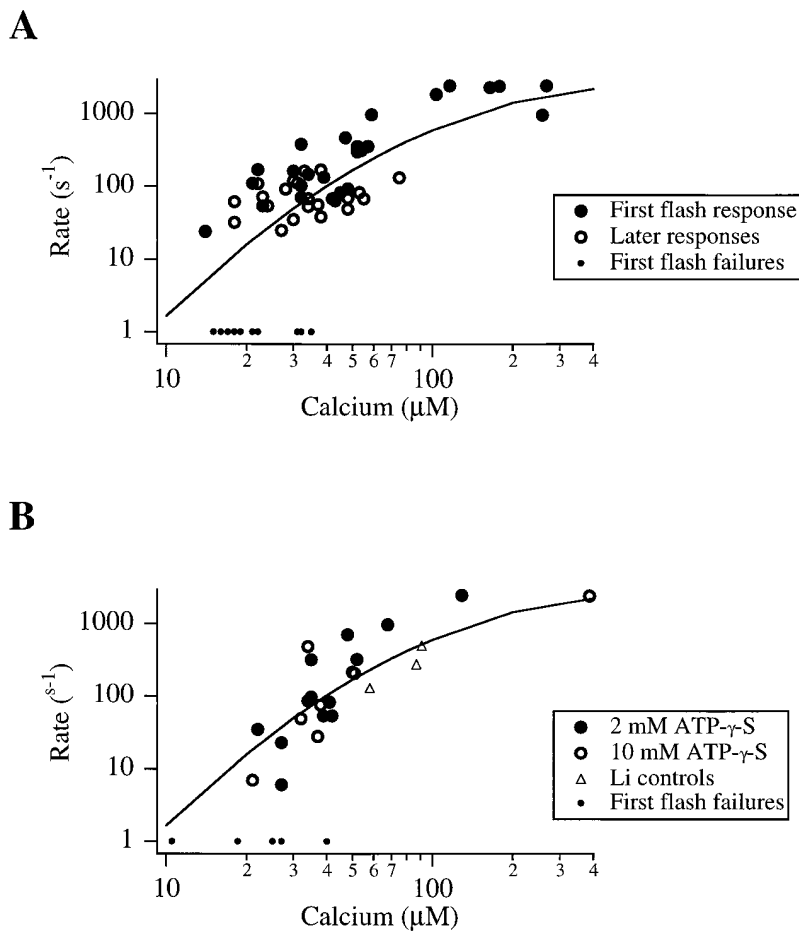
A

B

FIGURE 9. The calcium dependence of the rate of exocytosis in terminals with MgATP or ATP-γ-S. (*A*) The rate constant of the single exponential fit to the rising phase of each capacitance response in terminals dialyzed with MgATP (solution A) is plotted against the ratiometrically determined postflash intraterminal calcium concentration. (●) Data from the very first flash response. (○) Data from later flashes. No difference is noted between the two groups. The small, filled circles, plotted with an arbitrarily assigned rate constant of 1 s^{-1}, represent the postflash intraterminal calcium concentration that failed to evoke an increase in capacitance in a terminal that later responded to greater elevations of intraterminal calcium. To achieve these points, UV flashes were given with increasing intensity until the synaptic terminal responded. Only the maximum calcium concentration at which a terminal failed to respond is shown. The curve is drawn from our published model of secretion, which contains four sequential calcium-binding steps, followed by a final, calcium-independent fusion step (Heidelberger et al., 1994). Parameters used for the model calculation are identical to those previously described (Heidelberger et al., 1994). (*B*) The rate constant of the single exponential fit to the rising phase of the first capacitance response in terminals dialyzed with ATP-γ-S is plotted against the ratiometrically determined postflash intraterminal calcium concentration. (●) Data from terminals dialyzed with Solution C. (○) Data from terminals dialyzed with Solution B. No difference is noted between the two groups. (△) Data from terminals dialyzed with Solution E, a solution designed to control for the possible effects of Li$^+$ on exocytosis. The smaller symbols, plotted with an arbitrarily assigned rate constant of 1 s^{-1}, represent the postflash intraterminal calcium concentration that failed to evoke an increase in capacitance in a terminal without MgATP (solution B, C, or D) that later responded to a greater elevation of intraterminal calcium with a capacitance increase. As in *A*, the curve is drawn based upon our previously published model of secretion (Heidelberger et al., 1994).

ously published study (Heidelberger et al., 1994), in which no Mg^{2+} or nucleotide was added to the internal solution, were also binned and included for comparison (Fig. 10 *B*, □). At calcium concentrations >30 μM, the data are virtually superimposible, consistent with the interpretation that, in the calcium concentration ranges expected near release sites (Roberts et al., 1990; Llinas et al., 1992; Adler et al., 1991), cytosolic MgATP does not influence the kinetics of synaptic vesicle fusion of the release-ready pool. Similar observations have been made in neuroendocrine cells (Thomas et al., 1993). However, at calcium concentrations close to threshold, in the range 15–30 μM, interpretation of the data is less clear due to the small sample size, which is a function of the steepness of the rate versus calcium relationship and the complexity of the internal solution, which does not permit Ca^{2+} and Mg^{2+} to be independently controlled, and the inherent biological variability of the responses. From the few experiments that

were successfully conducted in the near-threshold range, a comparison of the rate constants from terminals dialyzed with the standard MgATP-containing solution, an ATP-γ-S-containing solution, and from previously published data, in which no Mg^{2+}, ATP, or ATP-γ-S was added to the internal solution, was made. In this near-threshold calcium range, terminals with MgATP exhibited a mean rate constant of 104 ± 29 s^{-1} (mean ± SEM, $n = 5$), terminals without Mg^{2+} or added nucleotide exhibited a mean rate constant of 77 ± 43 s^{-1} ($n = 5$), and terminals with ATP-γ-S exhibited a mean rate constant of 18 ± 7 s^{-1} ($n = 4$). While it appears that in this near-threshold calcium range the terminals with ATP-γ-S have a slower average rate than terminals with either MgATP or no added Mg^{2+} or nucleotide, both one-way ANOVA and Wilcoxon rank sums tests failed to demonstrate a difference among the three groups (ANOVA, $P = 0.22$; Wilcoxon, $P = 0.1$).

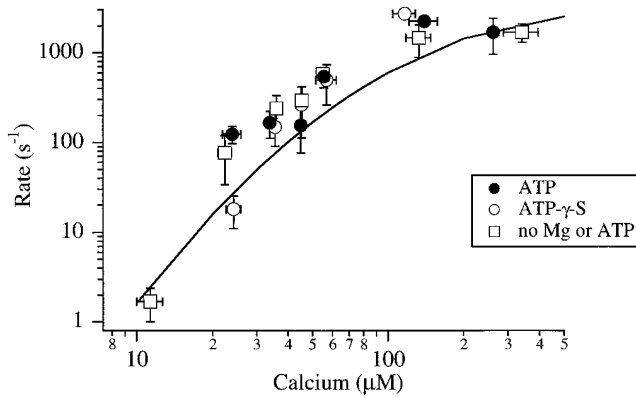Finally, the brief delay between the elevation of intra-

FIGURE 10. The calcium dependence of the rate of exocytosis may be independent of MgATP. Data are binned according to $[Ca^{2+}]_i$. The first bin is 0–14 μM, the second is 15–30 μM, the third is 31–40 μM, the fourth is 41–50 μM, and the fifth is 51–60 μM. At calcium concentrations >100 μM, bin sizes are somewhat larger. Each bin contains a minimum of two data points. Only the first flash responses are included. (●) Terminals with MgATP (solution A), (○) terminals with ATP-γ-S (solutions B or C), and (□) terminals with neither Mg^{2+} or nucleotide (solution D). Error bars represent ±1 SEM. The curve is based on our previously published model of the late stages of exocytosis in bipolar neurons, using the parameters described in Heidelberger et al. (1994).

terminal calcium and beginning of the capacitance rise was measured in terminals dialyzed with internal solution containing either MgATP or ATP-γ-S. This delay has been attributed to the binding kinetics of calcium to the calcium trigger for exocytosis (Heinemann et al., 1994). The delay was plotted against the intraterminal calcium concentration (Fig. 11 A). No difference in this relationship was detected between terminals with MgATP or ATP-γ-S. Even at near-threshold intraterminal calcium, there was no indication of a dependence upon MgATP. To provide a more complete comparison, data from our previously published study, in which no Mg^{2+} or nucleotide was added to the internal solution (Heidelberger et al., 1994), were binned according to the postflash intraterminal calcium and compared with binned data from the present study (Fig. 11 B). It can be seen that the average delays from all three sets of data show a similar relationship to postflash internal calcium. Furthermore, the relationship between the average delays and the binned intraterminal calcium concentration is equally well predicted for all three data sets by our published model (Heidelberger et al., 1994). These results are consistent with the inter-
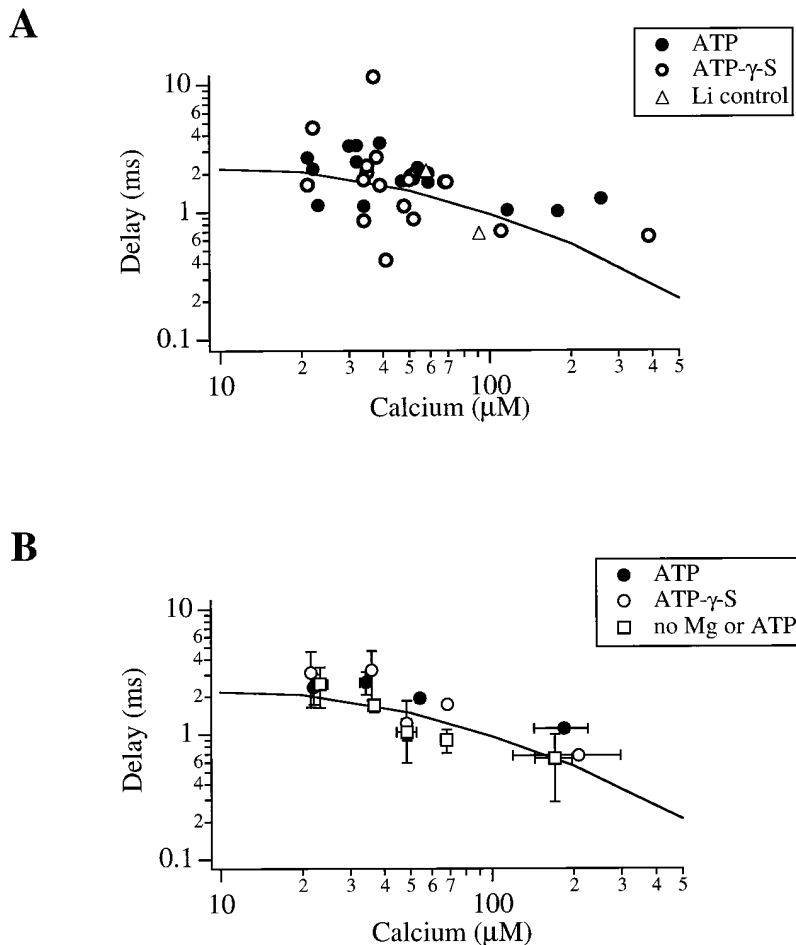
A

B

FIGURE 11. The delay between the rise in calcium and the start of the capacitance response is independent of MgATP. The delay is calculated as the time between the UV flash and the interception of the single exponential fit to the rising phase of the capacitance increase with the baseline. This delay was then corrected by 700 μs to take into account the delay introduced by the flash time course (0.6 ms) and the time (0.1 ms) required for calcium to be released from DM-nitrophen (Heidelberger et al., 1994; McCray et al., 1992). (●) Data from terminals dialyzed with MgATP (solution A). (○) Data from terminals dialyzed with ATP-γ-S (solution B or C). (△) Data from terminals loaded with Solution E, a solution designed to control for the possible effects of Li^+, which is contained in the ATP-γ-S solutions, on exocytosis. Data shown are from representative terminals that responded to elevation of $[Ca^{2+}]_i$ with a capacitance response that had a readily resolvable rising phase. Only first-flash responses are included. (B) Delays are binned according to $[Ca^{2+}]_i$ and expressed as mean ± 1 SEM. Each bin contains a minimum of 2 data points. (●) Binned data from terminals dialyzed with MgATP (solution A). (○) Binned data from terminals dialyzed with ATP-γ-S (solution B or C). (□) Binned data from terminals dialyzed with no added Mg^{2+} or nucleotide (solution D). The curve shown in A and B is drawn from our published model of secretion, which contains four sequential calcium-binding steps, followed by a final calcium-independent fusion step (Heidelberger et al., 1994).

pretation that the kinetics of calcium binding to the trigger for exocytosis are not significantly influenced by the concentration of cytosolic MgATP in synaptic terminals.

DISCUSSION

The Pool of Membrane that Contributes to the Capacitance Response Evoked by a Flash

A recent study has raised a concern about the validity of using flash photolysis of caged calcium in combination with capacitance measurements to study exocytosis (Oberhauser et al., 1995, 1996). This important issue was examined in the present study, and the results validate the combined use of these techniques in synaptic terminals of Mb1 bipolar neurons. First, it was shown that the pool of membrane that is able to fuse in response to the rapid and global elevation of calcium is specifically localized to the synaptic terminal. What synaptic-specific pool of membrane could be responsible for this increase in capacitance? The observed rise in capacitance, measured on the submillisecond time scale, is smooth rather than steplike (Figs. 5 *B* and 6 *B, insets*), consistent with the fusion of many small organelles, rather than a few large ones. EM analysis of Mb1 bipolar neuron synaptic terminals has revealed that terminals are packed with 500,000–900,000 small, clear core synaptic vesicles and contain a number of mitochondria and the rare endosome (von Gersdorff et al., 1996). Therefore, the most likely source of the synaptic-specific fusible pool of membrane is the abundant synaptic vesicle. This hypothesis is supported by the results of the cross-depletion study, which demonstrated that membrane depolarization, of a duration and intensity known to evoke glutamate release from bipolar neuron synaptic terminals (Tachibana and Okada, 1991; Tachibana et al., 1993), taps the same pool of membrane as a UV flash. The parsimonious interpretation, that in synaptic terminals of Mb1 bipolar neurons, flash photolysis of caged calcium triggers the fusion of synaptic vesicles with the plasma membrane, justifies the combined used of flash photolysis of caged calcium and capacitance measurements for the study of calcium-dependent exocytosis in this preparation.

The Maximum Rate of Exocytosis

Previously, we reported that the maximum rate of exocytosis saturated at calcium concentrations >200 μM with a rate constant of 2,000–3,000 s^{-1} (Heidelberger et al., 1994). This rather abrupt saturation of the rate constant became an important feature when modeling the calcium dependence of the rate of exocytosis and necessitated the incorporation of a cooperative interaction between the four proposed calcium binding sites.

One point that has required clarification is whether this saturation truly reflects a limiting rate of the secretory machinery or whether it might be due to another phenomenon. Bipolar neuron synaptic terminals make ribbon synapses (Dowling, 1987), and one might imagine, for example, that if fusion of the entire release-ready pool of vesicles at a synaptic ribbon required translocation of synaptic vesicles along the ribbon to release sites located near the base of the ribbon (Bunt, 1971; Dowling, 1987; von Gersdorff et al., 1996), then this translocation could require a source of energy such as might be provided by the hydrolysis of ATP. The results of the present study indicate that this is unlikely as neither the calcium concentration nor the value at which the rate saturates is affected by the presence or absence of cytosolic MgATP or a nonhydrolyzable ATP analog. Furthermore, the maximum rate constant measured in these flash-photolysis experiments is strikingly similar to the maximum rate constant for release of the recently described small ultrafast pool of synaptic vesicles (Mennerick and Matthews, 1996), supporting the idea that the value of 2,000–3,000 s^{-1} may indeed represent the maximum rate of the fusion machinery. An interesting question that then arises is how the entire release-ready pool of synaptic vesicles manages to fuse within 1 ms in response to flash photolysis of caged calcium. One possibility, discussed in von Gersdorff et al. (1996), is that the synaptic ribbon invaginates into the presynaptic membrane such that each vesicle tethered to the ribbon lies in close proximity to the plasma membrane (Raviola and Gilula, 1975; Fields and Ellisman, 1985; von Gersdorff et al., 1996) and is able to fuse laterally, rather than requiring a translocation step to the ribbon base. A second possibility, which we have raised before, is that of compound fusion (Alvarez de Toledo and Fernandez, 1990; Heidelberger et al., 1994). If compound fusion were the underlying mechanism, then vesicles would be predicted to fuse with each other at the same maximum rate at which an individual vesicle fuses with the plasma membrane in order for the maximum rates triggered by a flash to match those of the ultrafast pool. A recent study has reported that in addition to the presence of v-SNARES on synaptic vesicle membranes, synaptic vesicles also contain significant amounts of t-SNARES (Walch-Solimena et al., 1995), and it has been shown that these proteins can interact with one another in the absence of plasma membrane (Otto et al., 1997). It has also been recently shown that homeotypic fusion in the yeast vacuolar system involves the interaction of v-SNARES on one vacuole with the t-SNARES of another (Nichols et al., 1997). Thus, compound fusion could conceivably involve a mechanism similar to that of exocytosis. Regardless of the exact mechanism of synaptic vesicle fusion, the evidence points to a maximum fusion rate of ~2,000–

$3,000 \text{ s}^{-1}$, and, therefore, consideration of the abrupt saturation of the rate constant when modeling the calcium dependence of the rate of exocytosis is appropriate (Heidelberger et al., 1994).

*Is There a Requirement for Cytosolic MgATP
Late in Exocytosis?*

In the present study, the relationship between calcium and synaptic vesicle fusion was quantitatively examined and compared in terminals dialyzed with either MgATP or MgATP-γ-S. The relationship between the rate of synaptic vesicle and the intraterminal calcium concentration was little affected by the presence of cytosolic MgATP or ATP-γ-S at calcium concentrations >30 μM. With either nucleotide, the threshold for secretion was >∼20 μM, and the half-maximal rate of fusion was reached between 100 and 200 μM, consistent with exocytosis being a low affinity, high threshold process. The relationship between the measured delays and the intraterminal calcium also failed to reveal a dependency upon cytosolic MgATP. This was true at near-threshold calcium concentrations as well as at concentrations at which the rate constant saturated. Regardless of the nucleotide, the steep dependence of the rate of release upon calcium and the relationship between the measured delays and calcium was well fit by our previous model and is consistent with a Hill coefficient of 4 and a cooperative interaction between the calcium binding sites. The excellent concurrence between the present and former work emphasizes the strength of the fundamental relationship between the rate of exocytosis and calcium and the consistency of the results, despite some differences in methodology, but the results do not favor a role for cytosolic MgATP in exocytosis downstream of calcium elevation. One question not addressed in the present study is whether MgATP could be so tightly held within a protein complex such as the 20S complex that it is not readily removed or replaced by dialysis of the cytosol. In this scenario, the requirement for cytosolic MgATP would precede calcium elevation as described here, but the hydrolysis of ATP could occur at a later point in time.

*Is There a Cytosolic Requirement for MgATP
Early in Exocytosis?*

Secretion studies in neuroendocrine cells have also failed to reveal a requirement for cytosolic MgATP downstream of calcium entry (e.g., Parsons et al., 1995), but they have identified a requirement for MgATP at an upstream location (Holz et al., 1989; Bittner and Holz, 1992*a*; Hay and Martin, 1992) that may be associated with docking (Martin et al., 1995; Parsons et al., 1995). In the present study, an upstream role for MgATP was suggested by examining the number of times a terminal was able to respond to a superthreshold UV flash with a capacitance increase. Only terminals supplied with MgATP could undergo multiple rounds of release without significant decrement in response amplitude (Fig. 8). The nonhydrolyzable ATP-analog ATP-γ-S could not reproduce this effect. Additionally, in a study of paired-pulse depression in bipolar neuron synaptic terminals, MgATP was found to be required for brisk recovery, and this requirement could not be fulfilled with ATP-γ-S (Heidelberger and Matthews, 1997). These results indicate an important role for ATP hydrolysis in refilling of the release-ready pool of synaptic vesicles.

The reduction of the mean capacitance response to second and third flashes in terminals without MgATP was substantial (Fig. 8), and dialysis with millimolar ATP-γ-S, with and without magnesium chelation, produced complete blockade of the second capacitance response in approximately half of the terminals examined. It is not clear why some terminals were able give a second, typically small, capacitance response without MgATP; however, several possibilities exist. One is that these terminals may have produced sufficient endogenous MgATP to be able to compete with millimolar ATP-γ-S and/or magnesium depletion to permit a slow rate of pool refilling. A second possibility is that some of the vesicles primed by MgATP failed to fuse in response to the first stimulus, but fused in response to the second. This could occur if some synaptic vesicles have a higher calcium threshold for release than others or if synaptic vesicles must undergo additional maturation steps after the requirement for cytosolic MgATP is met to become fully fusion competent. The first of these two suggestions seems unlikely because a paired-pulse study in synaptic terminals has shown that a second pulse given immediately after the first fails to trigger any additional increase in membrane capacitance despite the further elevation of internal calcium (von Gersdorff and Matthews, 1997). Moreover, there is no discernible relationship between the amplitude of the capacitance response and global calcium (Fig. 7). However, it is plausible that at the time of the first flash, some synaptic vesicles could be independent of cytosolic MgATP without having achieved full fusion competence, but might become fusion competent by the second flash. Additional granule maturation steps after ATP-dependent priming have been proposed for neuroendocrine cells (e.g., Bittner and Holz, 1992*b*; Thomas et al., 1993). Further experiments will need to be performed to determine whether a similar maturational process occurs in synaptic terminals and whether this or another mechanism is responsible for the observed result.

Endocytosis is impaired in the absence of MgATP (e.g., Fig. 6; Heidelberger and Matthews, 1997) and,

therefore, another potential explanation for the lack of robust multiple responses in the absence of MgATP is that the inability to recycle vesicles impedes refilling of the release ready pool. Such a dependency of exocytosis on endocytosis has been reported at the neuromuscular junction (Koenig et al., 1989). Three lines of evidence suggest that this is not the case in retinal bipolar neurons. Firstly, Mb1 bipolar neuron synaptic terminals possesses an enormous pool of synaptic vesicles that could potentially be used to replenish the release-ready pool (von Gersdorff et al., 1996). Secondly, when fast endocytosis was inhibited by slight elevation of internal calcium, several rounds of exocytosis could be evoked reproducibly (von Gersdorff and Matthews, 1997). Thirdly, when endocytosis was inhibited by slight pressure-induced swelling that did not alter internal calcium, steplike capacitance increases of constant amplitude were observed with successive depolarizations (Heidelberger and Matthews, unpublished observations). These data argue against a tight linkage between endocytosis and the ability to respond to subsequent stimuli in the Mb1 bipolar neuron synaptic terminal. Given these observations, the data presented here are most consistent with a role for MgATP in preparing synaptic vesicles for fusion. The mechanism underlying the MgATP requirement is not yet known, but possibilities include a MgATP-dependent step in tethering vesicles to the synaptic ribbons or a MgATP-dependent priming step that might be mediated by hydrolysis of ATP.

Implications of MgATP-independent Release at a Ribbon Synapse

Two lines of evidence indicate that flash photolysis of caged calcium triggers the release of synaptic vesicles from a release-ready pool. The first is that the population of synaptic vesicles that has been physiologically defined to be the release-ready pool (von Gersdorff and Matthews, 1997), was shown in the present cross-depletion experiments to be the same pool of membrane that is triggered to fuse with the plasma membrane in response to a UV flash. Secondly, the rapid exocytotic response evoked by the first superthreshold UV flash is MgATP independent, suggesting that the pool of vesicles that fused in response to the flash had previously undergone the last ATP-dependent priming reaction. The subcellular localization of the release-ready pool of synaptic vesicles has not yet been directly determined; however, the excellent agreement between the number of vesicles tethered to the synaptic ribbons of an Mb1 bipolar neuron synaptic terminal and the amplitude of the capacitance response provides a strong indication that the release-ready pool of vesicles corresponds to those vesicles that are tethered to the synaptic ribbons (von Gersdorff et al., 1996). This premise is substantiated by the lack of other identifiable sites of vesicular release by EM criteria in the synaptic terminal (von Gersdorff et al., 1996). By analogy to conventional synapses, docked vesicles would be those that contact the plasma membrane. A single row of vesicles on either face of the ribbon was found to touch the plasma membrane in bipolar neuron synaptic terminals (von Gersdorff et al., 1996), and fusion of this pool of vesicles would be expected to contribute no more than 40 fF to the capacitance response, assuming a mean vesicle capacitance of 26.4 aF and 60 synaptic ribbons/terminal (von Gersdorff et al., 1996). However, the mean amplitude of the first flash response exceeds this limit, regardless of the nucleotide content of the internal solution. This raises the intriguing hypothesis that the last MgATP-dependent priming step may occur before vesicle docking at the plasma membrane in ribbon synapses of the retinal bipolar neuron. A similar suggestion has made been recently for the NSF-mediated ATP-dependent priming step in homeotypic fusion in yeast (Mayer et al., 1996) and in synaptic vesicle fusion (Otto et al., 1997). Future studies will need to be performed to determine whether this hypothesis is true for ribbon synapses of the bipolar neuron and for other central synapses.

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