

Roles of ATP in Depletion and Replenishment of the Releasable Pool of Synaptic Vesicles

RUTH HEIDELBERGER,¹ PETER STERLING,² AND GARY MATTHEWS³

¹*Department of Neurobiology and Anatomy and The W. M. Keck Center for the Neurobiology of Learning and Memory, University of Texas Medical School, Houston, Texas 77030;* ²*Department of Neuroscience, University of Pennsylvania, Philadelphia, Pennsylvania 19104;* and ³*Department of Neurobiology and Behavior, State University of New York, Stony Brook, New York 11794-5230*

Received 18 January 2002; accepted in final form 13 March 2002

Heidelberg, Ruth, Peter Sterling, and Gary Matthews. Roles of ATP in depletion and replenishment of the releasable pool of synaptic vesicles. *J Neurophysiol* 88: 98–106, 2002; 10.1152/jn.00039.2002. Synaptic terminals of retinal bipolar neurons contain a pool of readily releasable synaptic vesicles that undergo rapid calcium-dependent release. ATP hydrolysis is required for the functional refilling of this vesicle pool. However, it was unclear which steps required ATP hydrolysis: delivery of vesicles to their anatomical release sites or preparation of synaptic vesicles and/or the secretory apparatus for fusion. To address this, we dialyzed single synaptic terminals with ATP or the poorly hydrolyzable analogue ATP- γ S and examined the size of the releasable pool, refilling of the releasable pool, and the number of vesicles at anatomical active zones. After minutes of dialysis with ATP- γ S, vesicles already in the releasable pool could still be discharged. This pool was not functionally refilled despite the fact that its anatomical correlate, the number of synaptic vesicles tethered to active zone synaptic ribbons, was completely normal. We conclude 1) because the existing releasable pool is stable during prolonged inhibition of ATP hydrolysis, whereas entry into the functional pool is blocked, a vesicle on entering the pool will tend to remain there until it fuses; 2) because the anatomical pool is unaffected by inhibition of ATP hydrolysis, failure to refill the functional pool is not caused by failure of vesicle movement; 3) local vesicle movements important for pool refilling and fusion are independent of conventional ATP-dependent motor proteins; and 4) ATP hydrolysis is required for the biochemical transition of vesicles and/or release sites to fusion-competent status.

INTRODUCTION

The importance of ATP hydrolysis in calcium-triggered exocytosis is well established in neuroendocrine cells, where ATP has been shown to be required for one or more priming steps that are prerequisite for the rapid calcium-dependent fusion of dense-core secretory granules with the plasma membrane (Hay and Martin 1992; Holz et al. 1989; Parsons et al. 1995). Although ATP may act at a variety of steps in the exocytotic process, evidence suggests that ATP hydrolysis is required at early stages of granule recruitment and/or priming rather than at the final step of fusion itself. For instance, replacing ATP with nonhydrolyzable analogues does not affect the kinetics of fusion but does reduce the size of the release-

ready pool of granules in experiments using flash photolysis of caged Ca^{2+} to trigger exocytosis in adrenal chromaffin cells (Parsons et al. 1995; Xu et al. 1999). Taken together, these results indicate that ATP utilization is necessary to maintain secretory granules in the releasable pool but is not required for fusion. Heidelberg (1998) also reached similar conclusions based on experiments using flash photolysis of caged Ca^{2+} in synaptic terminals of retinal bipolar neurons, which release the neurotransmitter glutamate (Tachibana and Okada 1991; Tachibana et al. 1993) from small, clear-core synaptic vesicles.

Known ATP-dependent preparatory steps in exocytosis may be categorized into those that mobilize synaptic vesicles to the active zones and those that prepare the secretory apparatus for rapid, Ca^{2+} -dependent release. Physiological and biochemical approaches commonly used to detect neurotransmitter release do not distinguish between these categories; they simply report whether or not exocytosis has occurred. To discriminate between an ATP-dependent local vesicle trafficking step and an ATP-dependent preparatory step, it is also necessary to determine whether vesicles are correctly localized to active zones. Thus an assay of exocytosis should be combined with the visualization of active zones. In chromaffin cells of the adrenal gland, such a combined approach has indicated that the last ATP-dependent preparatory step for hormone exocytosis occurs near or at the time that a secretory granule contacts the plasma membrane (Parsons et al. 1995). In this study, we use a combined approach to characterize, for the first time, the nature of the last ATP-dependent step in Ca^{2+} -triggered glutamate release at a vertebrate central synapse.

Synaptic terminals of goldfish retinal bipolar neurons are amenable to a direct time-resolved presynaptic assay of exocytosis (von Gersdorff et al. 1998) and have active zones that are easily identified. In addition to the usual pre- and postsynaptic hallmarks of synapses, active zones of bipolar neurons are characterized by specialized structures called synaptic ribbons. Synaptic vesicles at ribbon-style active zones are tethered by filaments of uncertain composition to the ribbons; this suggests that a molecular motor may convey vesicles along the ribbons to sites of fusion (Bunt 1971; Lenzi and von Gersdorff 2001). Consistent with the idea that ribbons mark active zones,

Address for reprint requests: R. Heidelberg, Dept. of Neurobiology and Anatomy, UT-Houston Medical School, MSB 7.046, 6431 Fannin, Houston, TX 77030 (E-mail: ruth.heidelberg@uth.tmc.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

calcium channels, which are necessary for triggering exocytosis, are clustered at synaptic ribbons (Issa and Hudspeth 1994; Morgans 2001; Morgans et al. 2001; Raviola and Raviola 1982). Omega profiles indicative of vesicle fusion or fission have been identified beneath synaptic ribbons (Lenzi et al. 1999) in agreement with freeze fracture images in bipolar neurons indicating synaptic vesicle exocytosis at these sites (Raviola and Raviola 1982). In addition, following stimulation, there is a loss of vesicles from ribbons (Henry and Mulroy 1995; Lenzi et al. 2000). In bipolar neurons, synaptic ribbons tether some 5,000–6,000 vesicles in the terminal as a whole (von Gersdorff et al. 1996), and a similar number of vesicles can be released within a few hundred milliseconds upon activation of voltage-gated calcium channels. This correspondence suggests that the vesicles tethered to the ribbons constitute the releasable pool of synaptic vesicles (von Gersdorff et al. 1996).

In this study, we used a physiological stimulus to deplete the releasable pool of vesicles in bipolar neuron synaptic terminals that were dialyzed with internal solution containing either ATP or the poorly hydrolyzable analogue, ATP- γ S. After assessing whether this pool was functionally refilled, the active zones of the physiologically characterized terminals were then analyzed at the ultrastructural level to determine whether the anatomical correlate of the physiologically defined releasable pool was refilled. We found that although ATP hydrolysis is required for the functional replenishment of the releasable pool, it is not required for vesicles to physically repopulate ribbon-style active zones. Thus movement and attachment of vesicles from the cytosol to ribbons are unlikely to require ATP hydrolysis. Further, our data demonstrate that the last ATP-dependent preparatory step occurs after vesicles reach the active zone, pointing toward the biochemical maturation of synaptic vesicles and/or release sites as the last ATP-dependent step in neurotransmitter release. Given the placement of synaptic ribbons with respect to the plasma membrane, this maturational step may occur while a vesicle is tethered to a ribbon but before it contacts the plasma membrane.

METHODS

Cell isolation and solutions

Single bipolar neurons were isolated from goldfish retina as described (Heidelberger and Matthews 1992). Recordings were typically made from isolated synaptic terminals, which were selected on the basis of their characteristic appearance, size (8–12 μ m diam), and distinctive electrophysiological profile (von Gersdorff and Matthews 1994). The external solution consisted of (in mM) 115 NaCl, 2.5 KCl, 1.6 MgCl₂, 1 or 2.5 CaCl₂, 10 glucose, and 10 HEPES, pH = 7.4. The intracellular patch-pipette solution contained (mM): 130 Cs-gluconate, 10 TEA-Cl, 3 MgCl₂, 0.5 GTP, 5 EGTA, 2.5 CaCl₂, 0.1 fura-2, and 20 HEPES, pH = 7.2, with osmolarity adjusted to 279 ± 3 mosM. The internal solution also contained either ATP (2 mM Na₂ATP) or ATP- γ S (2 mM Li₄ATP- γ S). Control experiments showed that addition of 5 or 20 mM LiCl did not affect exocytosis, endocytosis, or pool refilling ($n = 4$). Internal calcium buffering was chosen to minimize increases in internal Ca²⁺ produced by ATP- γ S (Zenisek and Matthews 2000), which might have confounding effects on pool refilling. In experiments with 10 mM MgATP, the internal solution contained (in mM) 100 Cs-gluconate, 10 TEA-Cl, 2 MgCl₂, 0.5 EGTA, 35 HEPES, 10 MgATP, 0.5 GTP, and 0.2 fura-2. Because internal ATP is required to maintain the L-type presynaptic calcium current (Heidelberger and Matthews 1992; Tachibana et al. 1993),

omission of internal ATP or substitution with analogues such as AMP-PNP was not feasible.

Electrophysiological and [Ca²⁺]_i measurements

Whole cell recordings were performed at 21–24°C using 7- to 12-M Ω patch pipettes coated with silicone elastomer (Sylgard) or dental wax, and an EPC-9 patch-clamp amplifier controlled by E9screen or Pulse software (HEKA Elektronik, Lambrecht, Germany). Capacitance measurements were made using the automatic capacitance compensation of the EPC-9 amplifier or the software lock-in extension of the Pulse software (Gillis 1995). In the latter case, a 1,000- or 1,600-Hz sinusoidal stimulus with a peak-to-peak amplitude of 32 mV was superimposed on the holding potential of –60 mV. For measurement of [Ca²⁺]_i using fura-2, [Ca²⁺]_i was calculated from the ratio of the emitted light at two excitation wavelengths (Grynkiewicz et al. 1985), using calibration constants determined by dialyzing cells with highly buffered, known concentrations of Ca²⁺ (Heidelberger and Matthews 1992).

Electron microscopy

For electron microscopy, a synaptic terminal attached to a film of Aclar was recorded under patch clamp and then fixed by local superfusion with 2.5% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M phosphate buffer from an application pipette with a tip diameter of ~ 20 μ m that was placed within 15 μ m of the terminal. After 5 min, the bath fluid was replaced with fixative, a rectangular mark was etched into the Aclar film around the recorded cell using the superfusion pipette, and the dish was placed at 4°C overnight. Cells were then further fixed in 1% OsO₄ + 1.5% K ferrocyanide in 0.1 M phosphate buffer, dehydrated, and embedded in Embed 812. The embedded sample was peeled from the Aclar film, glued to an Epon blank, and sectioned serially at ~ 90 nm. The rectangular mark etched into the Aclar after recording left a corresponding mark in the cured embedding medium to guide sectioning. Sequences of 5–10 sections were mounted on formvar-coated slot grids, stained in methanolic uranyl acetate and lead citrate, and then photographed in an electron microscope (120 kV) at $\times 10,000$.

RESULTS

ATP is required to refill the releasable pool

Synaptic terminals of retinal bipolar neurons contain a limited pool of releasable neurotransmitter, which is exhausted within a few hundred milliseconds during depolarization (Menerick and Matthews 1996; Sakaba et al. 1997; von Gersdorff and Matthews 1994; von Gersdorff et al. 1998). Once depleted, the releasable pool refills slowly, resulting in paired-pulse depression lasting several seconds (von Gersdorff and Matthews 1997). We examined whether ATP hydrolysis is required for replenishment of the releasable pool in bipolar cells, using capacitance measurements from single synaptic terminals as an index of transmitter release. To determine the rate of refilling of the releasable pool, a depolarization sufficient to exhaust the pool was applied, followed a variable time later by a second strong depolarization to test the state of the pool. Refilling of the releasable pool in terminals dialyzed with internal solution containing 2 mM ATP is illustrated in Fig. 1A (●), which shows that recovery proceeded exponentially with a time constant of 6.5 s under these experimental conditions (solid line). On average in terminals with 2 mM ATP, the second capacitance response was $105 \pm 5\%$ (mean \pm SE; $n = 19$) of the first when the interval between stimuli was > 20 s.

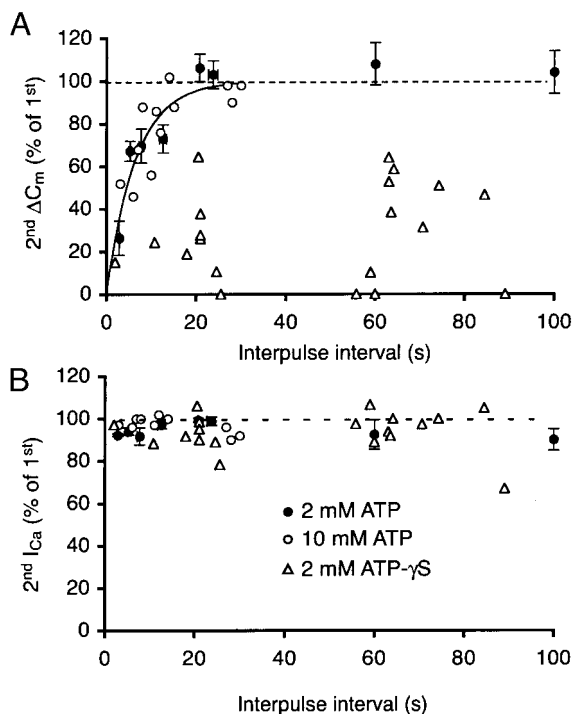


FIG. 1. Recovery from paired-pulse depression in synaptic terminals dialyzed with internal solution containing ATP or ATP- γ S. The legend in B also applies to A. Individual data points for experiments with 10 mM ATP (\circ) or 2 mM ATP- γ S (Δ) represent results from single synaptic terminals. Data points for experiments with 2 mM ATP (\bullet) represent averages from 5 to 13 synaptic terminals (± 1 SE). A: a 1st pulse sufficient to exhaust the releasable pool was followed after a variable interpulse interval by a 2nd depolarization. Full recovery of the capacitance response required ~ 20 s in synaptic terminals recorded with ATP-containing internal solution. Solid line, an exponential function with a time constant of 6.5 s fitted to the results with 2 mM internal ATP by a least-squares criterion. Recovery was incomplete in cells recorded with ATP- γ S even after much longer interpulse intervals. B: calcium current was minimally depressed by the conditioning depolarization, and there was no difference among the 3 experimental conditions.

Thus in the presence of ATP, ~ 20 s between successive stimuli was sufficient for complete recovery of a maximal depolarization-evoked capacitance response.

We next repeated this experiment but replaced ATP in the internal solution with its poorly hydrolyzed analogue, ATP- γ S. As shown in Fig. 1A (triangles), pool replenishment was slow and incomplete in the presence of ATP- γ S. For example, with an interpulse interval of ~ 20 s, the second capacitance response averaged $27 \pm 8\%$ of the initial response ($n = 7$), compared with $105 \pm 5\%$ in the presence of ATP. Impaired replenishment of the releasable pool by ATP- γ S cannot be explained by failure of the presynaptic calcium current to recover from any inactivation induced by the first pulse of a pair. Figure 1B shows that calcium current was only slightly depressed after the initial stimulus and did not change significantly at all interpulse intervals regardless of whether the internal solution contained ATP or ATP- γ S. Like ATP, ATP- γ S supports normal function of calcium channels, presumably by serving as a substrate for thiophosphorylation of the channels by kinases. As a result, there is no significant paired-pulse depression of calcium current. By contrast, ATP- γ S significantly reduces functional refilling of the releasable pool of vesicles, possibly by interfering with ATP hydro-

lysis by ATPases that are required to prepare vesicles for rapid calcium-triggered exocytosis.

The finding that ATP hydrolysis is required for pool refilling raises the possibility that ATP availability may also limit the rate of recovery from paired-pulse depression in cells recorded with an internal solution containing 2 mM ATP. To examine this possibility, we increased the ATP concentration in the internal solution from 2 to 10 mM. Figure 1A (\circ) shows that the rate of pool replenishment after depletion was not affected by elevating the ATP concentration. Thus functional refilling of the releasable pool is already not limited by ATP availability at an internal concentration of 2 mM. The presynaptic calcium current was also not affected by raising the internal ATP concentration (Fig. 1B, \circ).

Inhibition of ATP hydrolysis did not affect the initial size of the readily releasable pool of synaptic vesicles

ATP- γ S did not affect the size of the initial capacitance response evoked by the first depolarization after beginning whole cell recording. Figure 2A shows that the average amplitude of the initial capacitance response in terminals recorded with 2 mM ATP- γ S was 189 ± 25 fF ($n = 12$), compared with an average initial response of 180 ± 17 fF ($n = 9$) in control terminals recorded with 2 mM ATP. Although the initial response was normal in the presence of ATP- γ S, subsequent responses were dramatically smaller (Fig. 2A) even though stimuli were spaced at intervals sufficient for full recovery in terminals dialyzed with ATP. As Fig. 2B demonstrates, the fall in exocytosis after the initial stimulus in terminals recorded with ATP- γ S was not associated with rundown of the calcium current, which remained constant in response to successive depolarizations over the course of the experiment. A simple interpretation of the result shown in Fig. 2A is that all of the vesicles in the releasable pool at the start of the experiment had already passed through any preparatory steps that require ATP hydrolysis. Hence, the presence of ATP- γ S did not affect their subsequent exocytosis in response to the initial depolarization. Once the preexisting group of vesicles was released, however, new vesicles could not be recruited into the releasable pool without ATP hydrolysis.

If vesicles already in the releasable pool at the start of whole cell recording undergo significant turnover even in the absence of a depolarizing stimulus, then the size of the capacitance response evoked by the initial depolarization should decline with time after the onset of dialysis with ATP- γ S. To examine this possibility, we measured the initial capacitance response at various times after break-in in a number of terminals dialyzed with ATP ($n = 9$) or ATP- γ S ($n = 12$). The results are summarized in Fig. 2C. The rise of fluorescence as fura-2 diffused into the terminal from the patch pipette (along with ATP- γ S or ATP) suggested that dialysis was complete within 20–30 s after breaking in to begin whole cell recording. However, the amplitude of the initial capacitance response did not change consistently with waiting times of ≤ 200 s during dialysis of synaptic terminals with either ATP or ATP- γ S. Thus we conclude that ATP- γ S did not disrupt the releasability of the preexisting set of vesicles in the rapidly releasable pool at least on a time scale of a few minutes. This behavior suggests that there was little resting turnover of the vesicles in the releasable pool in the absence of depolarization.

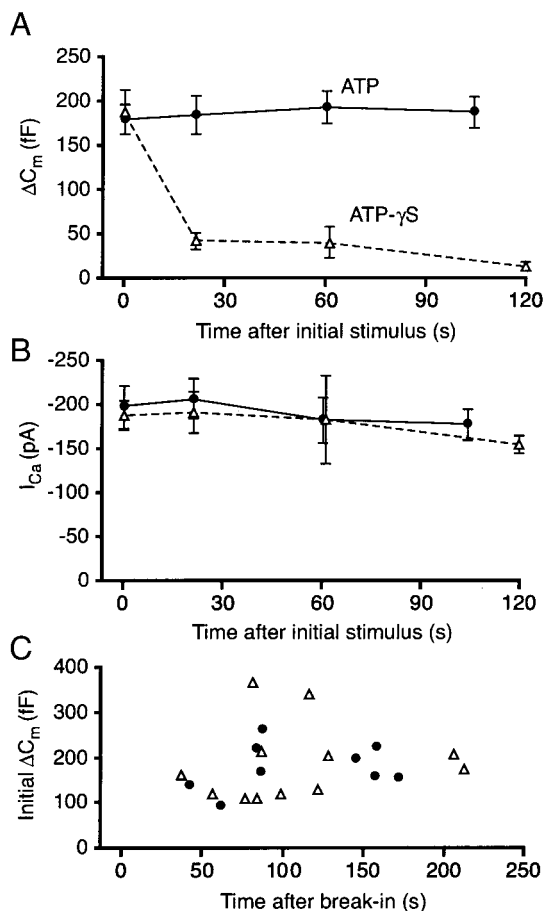


FIG. 2. Inhibition of ATP hydrolysis does not affect the size of the initial capacitance response in synaptic terminals but reduces subsequent responses. *A*: the average capacitance response to successive depolarizations in synaptic terminals dialyzed with internal solution containing 2 mM ATP (●) or 2 mM ATP- γ S (Δ). Each data point represents the average response from 5 to 12 synaptic terminals (± 1 SE). Time is expressed relative to the initial depolarization, which in each cell was the 1st stimulus presented after beginning whole cell recording. *B*: the average calcium current elicited by the same depolarizations shown in *A*. *C*: the size of the initial capacitance response as a function of dialysis time. Each symbol shows the result from a single bipolar cell synaptic terminal. In different cells, the initial depolarizing stimulus was presented at variable times after the beginning of whole cell recording (break-in). ●, terminals recorded with internal solution containing 2 mM ATP; Δ , terminals recorded with 2 mM ATP- γ S.

Vesicle docking at synaptic ribbons is unaffected by ATP- γ S

Active zones of bipolar-cell synaptic terminals are marked by distinctive synaptic ribbons, which are plate-like or spherical structures to which numerous synaptic vesicles are tethered by fine filaments. Ribbons are aggregates of the protein ribeye (Schmitz et al. 2000) and are separated from the plasma membrane at the active zone by a gap bridged by a filamentous complex that possibly includes the cytomatrix proteins bassoon and/or piccolo (Brandstatter et al. 1999; Dick et al. 2001). Vesicles tethered to the ribbons are thought to fuse with the plasma membrane during depolarization after being released from the ribbon or transported to the base of the ribbon. In this scenario, a simple explanation of the inhibition of pool refilling by ATP- γ S is that ATP hydrolysis is required for the movement and/or attachment of synaptic vesicles to the ribbons in the bipolar-cell synaptic terminal. In the presence of ATP- γ S, then, vesicles that were released from the ribbon and fused

with the plasma membrane during a prior depolarization might not be replaced by new vesicles, producing impaired refilling of the releasable pool as a result of the physical absence of vesicles. To investigate whether vesicles are physically absent from the ribbon in the presence of ATP- γ S, we used electron microscopy to examine the state of the synaptic ribbons in synaptic terminals that were recorded using intracellular solutions containing ATP or ATP- γ S. Two depolarizations were presented, separated by sufficient time for pool refilling under control conditions. The first depolarization depleted the releasable pool, while the second depolarization probed the state of this pool and confirmed the inhibitory effect of ATP- γ S on functional pool refilling. Then, the terminal was fixed by application of aldehyde fixatives ~ 30 s after the second stimulus and prepared for electron microscopy.

Figure 3 shows single sections through each of two terminals parallel to their sites of contact with the substrate, where the flattening of the membrane permits convenient observation of multiple ribbons in a single section plane. One terminal was recorded with internal solution containing ATP (*top left*) and the other with ATP- γ S (*bottom left*). Each section contains multiple ribbons, and each ribbon bears a halo of vesicles, which are shown at higher magnification on the *right*. No consistent differences were observed in the appearance of ribbons or vesicles between ATP-containing and ATP- γ S-containing internal solutions. Ribbons were fully populated with vesicles in both conditions, and filaments connecting vesicles to ribbons were observed in the presence of ATP or ATP- γ S.

To quantify the vesicle populations associated with synaptic ribbons, we counted the number of vesicles within 60 nm of the edge of a ribbon in sections from seven terminals with ATP and seven terminals with ATP- γ S. Examples of serial sections taken approximately parallel to the face of plate-like ribbons are illustrated in Fig. 4, which again shows no discernible difference between ATP and ATP- γ S. Figure 5 shows the histograms of the vesicle counts from single and serial sections in synaptic terminals recorded with ATP- γ S and in control cells with ATP. There was considerable overlap between the two distributions, and the average number of vesicles per ribbon in a single section was 21.5 ± 0.5 (mean \pm SE; $n = 209$) for ATP and 25.7 ± 0.7 ($n = 141$) for ATP- γ S. Although slight, this difference is statistically significant ($P = 5.6 \times 10^{-7}$; 2-tailed t -test). Thus failure of vesicles to populate ribbons cannot account for the inhibition of pool refilling by ATP- γ S. This result suggests that ATP hydrolysis is required to prepare vesicles for fusion at a step other than movement to and/or attachment to the ribbon.

DISCUSSION

Differential effects of ATP- γ S on release and refilling of the release-ready vesicle pool

Synaptic terminals of retinal bipolar neurons contain a discrete releasable pool of synaptic vesicles that undergo rapid, calcium-dependent fusion upon the activation of presynaptic voltage-gated calcium channels. We examined the role of ATP in the depletion and refilling of this well-defined pool. Our capacitance records demonstrate that the substitution of ATP- γ S for ATP in the internal solution had no effect on

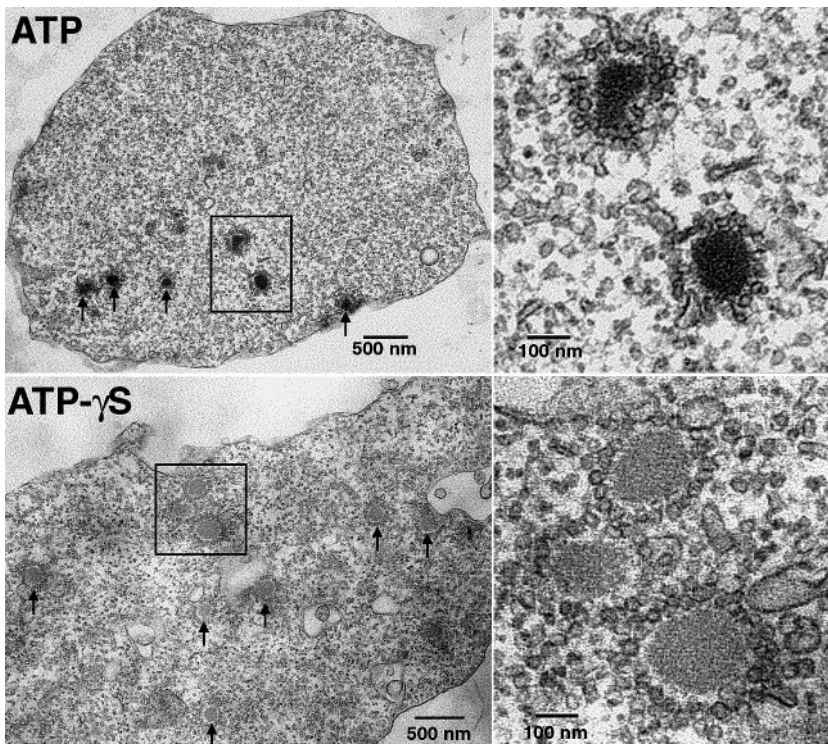


FIG. 3. ATP hydrolysis is not required for vesicles to repopulate the synaptic ribbons following a maximum capacitance jump. *Left*: low magnification electron micrographs of sections through terminals fixed ~ 30 s after a pair of pool-depleting depolarizations were given. *Top* and *bottom* cells, respectively, contained ATP and ATP- γ S. *Right*: ribbons outlined by each square are shown at higher magnification. Number of tethered synaptic vesicles that form the "halo" are similar. Difference in electron density of the ribbons in the 2 terminals reflects an unidentified but non-specific staining difference, which appeared with equal frequency in both conditions.

exocytosis triggered by the first pool-depleting stimulus. By contrast, ATP- γ S inhibited exocytosis in response to a second and subsequent stimuli; at best, even when the depolarizations were separated by more than 10 times the normal time constant of pool refilling, only partial refilling of the releasable pool occurred. This partial refilling may be due to the synthesis of

sufficient endogenous ATP to be able to compete with exogenous ATP- γ S to support a slow rate of pool refilling. Alternatively, the small number of vesicles that fused in response to a second stimulus in ATP- γ S terminals may represent vesicles that had already undergone the last ATP-dependent preparatory step at the time of the first pulse but were either too far from the active zone or required an additional priming step to achieve full fusion-competence. Our results agree with previous suggestions that ATP hydrolysis is required for preparatory phases of synaptic exocytosis but not for membrane fusion (Heidelberger 1998; Kawasaki et al. 1998; Schweizer et al. 1998). A unusual feature of the present study is that we had direct access to the isolated synaptic terminal and were able to monitor both intraterminal calcium (not shown) and presynaptic calcium current (see also Heidelberger

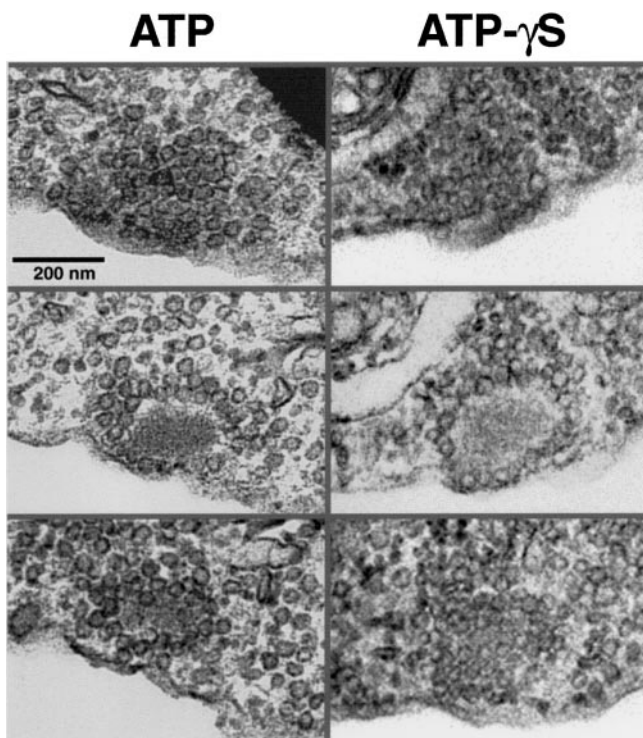


FIG. 4. Serial sections through terminals treated with ATP (*left*) or ATP- γ S (*right*) show similar numbers of vesicles docked at the plasma membrane and tethered to both faces of the ribbon.

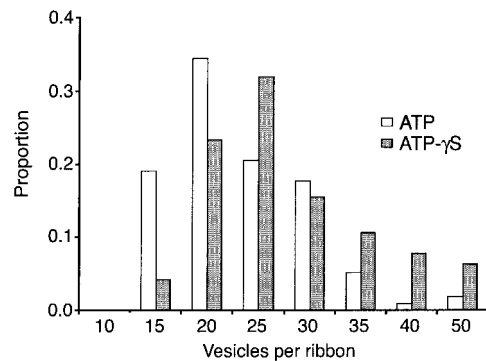


FIG. 5. Histograms of the number of synaptic vesicles associated with synaptic ribbons in the presence of ATP (\square) or ATP- γ S (\blacksquare). The number of vesicles per ribbon (abscissa) represents the number of vesicles within 60 nm of the ribbon in a single section not the total number of vesicles associated with the entire ribbon. The ordinate shows the proportion of the total number of counts falling within each bin. Vesicles were counted in 209 and 141 sections of ribbons in terminals recorded with ATP or ATP- γ S, respectively.

2001). Our data show that ATP- γ S inhibits functional refilling of the releasable pool of synaptic vesicles by acting at a locus other than resting calcium, calcium handling, or calcium entry.

ATP-dependent entry of a synaptic vesicle into the releasable pool is not readily reversible

ATP- γ S did not block the first round of release even after several minutes of dialysis. This observation demonstrates that ATP- γ S does not slowly or directly inhibit exocytosis of vesicles already in the releasable pool, lending further support for a role for ATP hydrolysis in refilling. Perhaps more importantly, the constancy of the pool size indicates that there is little turnover of the releasable pool at rest. Thus under our experimental conditions, ATP hydrolysis is not required for maintenance of the fusion-competent state of synaptic vesicles. By contrast, the continued presence of ATP is required to maintain the primed state of dense-core secretory granules (Xu et al. 1998, 1999). Indeed, 5 min of internal dialysis with a nonhydrolyzable ATP analogue such as ATP- γ S can completely block exocytosis of dense-core granules in adrenal chromaffin cells (Xu et al. 1999), whereas 3 min of dialysis in synaptic terminals does not. Three minutes should be more than sufficient for complete dialysis of synaptic terminals, given their smaller volume relative to adrenal chromaffin cells and the speed at which fura-2, which has a larger molecular weight than ATP- γ S, equilibrates within the terminals (see Pusch and Neher 1988). Therefore a potentially significant difference in the role of ATP in the maintenance of the fusion-competent state may exist between a neuroendocrine cell of the adrenal gland and a glutamatergic synaptic terminal of the CNS.

One ATP-dependent process that has been implicated in Ca^{2+} -triggered exocytosis of dense-core granules is phosphorylation of phosphatidylinositol to form PIP_2 (Hay and Martin 1993; Hay et al. 1995). An important effector of this PI kinase pathway, CAPS (calcium-dependent activator protein for secretion) (Loyet et al. 1998), is found on dense-core granules and is essential for dense-core granule exocytosis (Berwin et al. 1998; reviewed in Klenchin and Martin 2000). However, CAPS is not found on synaptic vesicles (Berwin et al. 1998), and it is not required for synaptic glutamate exocytosis (Rendon et al. 2001; Tandon et al. 1998). Thus the difference in requirement for ATP for maintenance of the releasable pool could reflect the type of secretory vesicle undergoing exocytosis, with dense-core granules, but not synaptic vesicles, requiring continual ATP hydrolysis by PI kinases for interaction with CAPS. Alternatively, a component of a shared ATP-dependent preparatory pathway may be modified such that the kinetics of exit from the releasable pool are faster in chromaffin cells or ATP- γ S is less effective as a substrate. That these two types of secretory cells, with their well-documented differences in the Ca^{2+} dependence of release (reviewed in Heidelberger 2001b) and the role of CAPS protein in exocytosis (Rendon et al. 2001; Tandon et al. 1998), might also have different ATP requirements for maintaining the releasable pool is a congruous finding. Further experiments are needed to more fully appreciate the mechanisms that govern the stability of the releasable pool at synapses.

Vesicles on ribbons are fully primed for exocytosis

The number of vesicles in the readily releasable pool in a bipolar cell terminal agrees quantitatively with estimates of the total number of vesicles tethered to synaptic ribbons (von Gersdorff et al. 1996). Because ATP- γ S did not affect the size of the initial releasable pool, we suggest that an important function of the synaptic ribbon may be to hold a pool of fusion-competent vesicles in readiness. Most vesicles associated with ribbons are far from the plasma membrane (see Figs. 3 and 4), which is the presumed locus of fusion. If vesicles tethered to synaptic ribbons in fact represent the releasable pool, then the last ATP-dependent priming step in the synaptic vesicle secretory pathway must occur prior to vesicle attachment at the plasma membrane. In this respect, our results are similar to those obtained with soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptor (SNARE)-mediated fusion of yeast vacuoles, where ATPase activity mediated by NSF and soluble NSF attachment protein (SNAP) is required only before the vacuoles are mixed (Ungermann et al. 1998). By contrast, NSF is thought to act after vesicle docking at the plasma membrane in secretory cells (e.g., Kawasaki et al. 1998; Parsons et al. 1995; Schweizer et al. 1998; Tolar and Pallanck 1998). Our results, along with those of Heidelberger (1998), raise the possibility that vesicles attached to the synaptic ribbon may serve as an alternative location for the last ATP-dependent preparatory step (Fig. 6A).

ATP-hydrolysis is required for preparing synaptic vesicles for fusion

A key feature of this study is that in addition to physiologically examining the requirement for ATP in the functional refilling of the releasable pool of synaptic vesicles, we also examined the role of ATP in the physical replenishment of this pool. Terminals with ATP exhibited the ability to both functionally and physically replenish the releasable pool. This is not unanticipated given that the onset of fixation was ~ 30 s after the second stimulus, which is sufficient time to allow for complete functional pool refilling (Fig. 1) (also von Gersdorff and Matthews 1997). Importantly, we found that the number of synaptic vesicles tethered to the ribbon-style active zones in terminals dialyzed with ATP- γ S did not reveal a deficit when compared with terminals with ATP. If anything, there were slightly *more* vesicles tethered to ribbons in ATP- γ S terminals, consistent with there being little spontaneous fusion or detachment of vesicles from ribbons in terminals with ATP- γ S relative to controls. Furthermore, there was no noticeable difference in the appearance of these synaptic vesicles between terminals with ATP or ATP- γ S (Figs. 3 and 4).

Because most synaptic vesicles associated with ribbons do not contact the plasma membrane, we do not believe that these vesicles represent fused vesicles that have not yet been retrieved. Nor is it likely that they represent newly retrieved vesicles because while ATP supports compensatory endocytosis, this type of endocytosis is inhibited by ATP- γ S (Heidelberger 2001a). The simplest interpretation is that in both groups, the synaptic vesicles on the ribbons represent new arrivals. Yet the physiology demonstrates that following depletion of the releasable pool, subsequent exocytosis occurs only when ATP is provided. Together, our results suggest that

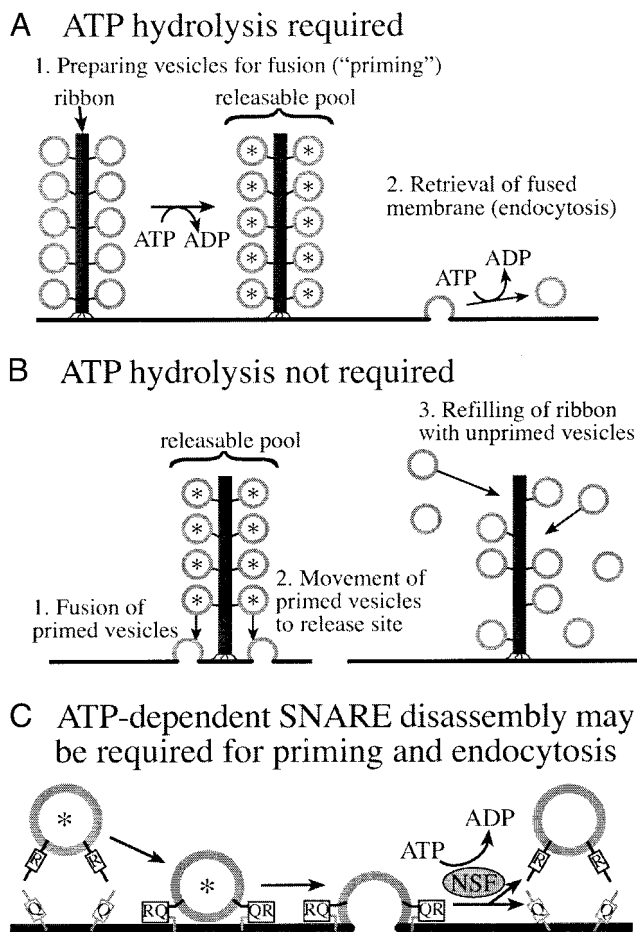


FIG. 6. ATP hydrolysis is required at specific steps along the secretory pathway. *A*: ATP hydrolysis is required for preparing vesicles for Ca^{2+} -dependent fusion and for postfusion membrane retrieval (endocytosis). ATP hydrolysis renders vesicles tethered to the synaptic ribbon fusion-competent. These comprise the releasable pool and are marked (*). Although ATP-dependent preparatory steps are depicted here as occurring on vesicles, ATP hydrolysis may also prepare fusion sites for release. *B*: ATP hydrolysis is not required for the fusion of vesicles in the releasable pool. Nor does it seem to be necessary for the movement of primed vesicles to sites of fusion or for the replenishment of the synaptic ribbon. *C*: a possible model to explain the dual actions of ATP- γ S on pool refilling and endocytosis. A primed vesicle with free R-SNAREs binds to Q-SNARE targets on the plasma membrane. Following fusion, the core complex is disassembled by ATPase activity of a protein such as NSF, freeing Q-SNAREs so that they may participate in the next round of release. The disassembly and sorting of SNARE complex also permits the fused membrane to enter the endocytic pathway. Note that there may be additional steps in the endocytic pathway following SNARE complex disassembly that are not depicted here. Some of these may require additional nucleotide hydrolysis.

the failure to functionally refill the releasable pool of synaptic vesicles in the presence of ATP- γ S, as demonstrated by capacitance measurements, is not due to the physical absence of synaptic vesicles at active zones. Rather failure of functional pool refilling in the presence of ATP- γ S represents a biochemical deficit.

A simple interpretation is that an ATPase requires energy released by ATP hydrolysis to establish molecular configurations necessary for fusion. For example, ATPase-activity of NSF may be required to disassemble SNARE protein complexes formed within the vesicle membrane (*cis*-SNAREs) and to make SNAREs on vesicles in the pool competent to interact

with partner proteins in the plasma membrane during calcium-triggered exocytosis (Bock et al. 2001; Hanson et al. 1997a,b; Hay and Scheller 1997; Otto et al. 1997). In addition, individual Q-SNAREs on the plasma membrane may relax into an inactive conformation that requires NSF ATPase-activity to interact with R-SNAREs on the vesicle membrane (Jahn and Südhof 1999) (see Fig. 6C). Alternatively, ATP may be required for a preparatory reaction step that involves a kinase. Protein kinases as well as PI kinases (Hay and Martin 1993; Hay et al. 1995) have been implicated in Ca^{2+} -triggered exocytosis. While ATP- γ S is a good substrate for many protein kinases, it is not known whether it can be used by PI kinases. If ATP- γ S fails to serve as a substrate for PI kinases, then lack of PIP_2 could potentially explain the action of ATP- γ S on the functional refilling of the releasable pool.

No role for conventional ATP-dependent motors in local vesicle movements

Motor proteins that utilize ATP as an energy source have been localized to synapses, including ribbon synapses. Brain myosin V is expressed in rod photoreceptor terminals and also in puncta in the inner plexiform layer that may correspond to sites within bipolar terminals (Schlamp and Williams 1996). The kinesin motor KIF3A is associated with synaptic vesicles near the ribbon and also with the ribbon itself (Muresan et al. 1999). In sympathetic and hippocampal neurons, myosin has been implicated in vesicle mobilization into releasable pools (Mochida et al. 1994; Ryan 1999). A local role for kinesins in synaptic function has not been established. However, a model developed for sea urchin eggs suggests that vesicles may be transported via kinesin/microtubules for the distance leg of the journey to the plasma membrane (Bi et al. 1997; see also review by Sokac and Bement 2000).

Movement powered by conventional kinesins and myosins requires ATP hydrolysis, and ATP- γ S is a poor substrate for these motors (Dantzig et al. 1988; Romberg and Vale 1993; Shimizu et al. 1991). Nevertheless, introduction of 2 mM ATP- γ S into a terminal did not affect the initial size of the secretory response or the attachment of vesicles to the synaptic ribbon (Figs. 3 and 4). Therefore if the releasable pool is composed of vesicles tethered to synaptic ribbons (von Gersdorff et al. 1996) and the tethered vesicles in our ultrastructural studies represent new vesicles, then arrival at and attachment to a synaptic ribbon must occur without assistance from such conventional motors (Fig. 6B). Similarly, because the first exocytotic response is normal in terminals with ATP- γ S, it is unlikely that conventional ATP-dependent motor proteins are required to move a vesicle on a ribbon to its presumed fusion site at the plasma membrane (Fig. 6B).

Unconventional motor proteins such as myosin V cannot be excluded as candidates for powering local vesicle movements on the basis of our data alone because it is not known whether these actin-based ATPases utilize ATP- γ S. However, it is interesting to note that even after prolonged stimulation, myosin light chain kinase inhibitors fail to completely prevent pool refilling (Ryan 1999). In addition, pool refilling at hippocampal synapses is completely normal in myosin Va mutant mice (Schnell and Nicoll 2001). This raises the possibility that there is more than one myosin or mechanism for mobilizing

vesicles to the active zones at conventional glutamatergic central synapses. The fact that actin depolymerization also has no effect on refilling of the releasable pool at hippocampal synapses (Morales et al. 2000) suggests that pool refilling from a local reserve may be independent of all myosin/actin interactions. We therefore speculate that the replenishment of ribbon-type active zones in retinal bipolar neurons may involve a local translocation step that is not powered by a myosin motor (Fig. 6B). However, whether unconventional myosins are involved or whether another motor protein or free diffusion is at play, are important questions that warrant further investigation.

Inhibition of compensatory endocytosis does not account for the loss of pool-refilling

Endocytosis was also impaired in terminals dialyzed with ATP- γ S (data not shown) (also Heidelberger 2001a). One interpretation is that impairment of vesicle retrieval may lead to a decrease in the number of vesicles available for refilling the releasable pool. However, several lines of evidence argue against this interpretation for the present study. The first is that, unlike at a conventional synapse, the releasable pool of synaptic vesicles comprises only a small percentage of the total synaptic vesicles present in a bipolar neuron synaptic terminal. Indeed, for each vesicle on a synaptic ribbon, there are ≥ 100 vesicles in the cytoplasmic pool that could potentially take its place (von Gersdorff et al. 1996). Second, blockade of endocytosis by other mechanisms does not prevent normal refilling and release in these terminals (von Gersdorff and Matthews 1997). Thus if inhibition of endocytosis by ATP- γ S is linked to the loss of functional pool refilling, the mechanism must be something other than the simple lack of membrane retrieval. Finally, our data show that ribbon-type active zones are replete with synaptic vesicles following functional depletion in the presence of ATP- γ S (Figs. 3 and 4) even though endocytosis was absent. Together these observations indicate that the physical replenishment of the releasable pool in our experiments occurs from sources other than recently recycled vesicles. The most obvious source is the several hundred thousand preformed synaptic vesicles that fill each terminal (von Gersdorff et al. 1996).

Requirement for ATP hydrolysis by NSF may influence both pool refilling and endocytosis

Although failure to retrieve membrane in and of itself may not account for the inhibition of pool refilling by ATP- γ S, the role of ATP hydrolysis, via NSF and SNAPs, in disassembly of SNARE complexes and activation of SNARE proteins suggests a scenario in which the dual actions of ATP- γ S on pool refilling and endocytosis may arise from a common molecular target (Fig. 6C). In this model, similar to the model recently proposed for *Drosophila* neurons (Littleton et al. 2001), SNARE core complexes formed between R-SNAREs on synaptic vesicles and Q-SNAREs on the plasma membrane must be disassembled after Ca^{2+} -triggered vesicle fusion and sorted prior to endocytosis. Disassembly of these core complexes and recycling of the SNAREs requires ATP hydrolysis by NSF. By inhibiting the disassembly of the core complex (Hanson et al. 1997b), ATP- γ S may simultaneously prevent the refilling of depleted vesicle pools with fusion competent synaptic vesicles

and the entry of fused membrane into the endocytic pathway. Even if new vesicles are available at the active zone as we suggest and even if those vesicles possess fusion-competent R-SNARE components, failure of disassembly of SNARE complexes from vesicles that previously fused at the active zone may limit the availability of Q-SNARE partners.

We thank Dr. Jian Li for preparing the electron micrographs.

This work was supported by National Eye Institute Grants EY-12128 (to R. Heidelberger), EY-00828 (to P. Sterling), and EY-03821 (to G. Matthews) and by the Esther A. and Joseph Klingenstein Fund (to R. Heidelberger) and the Alfred P. Sloan Foundation (to R. Heidelberger).

REFERENCES

- BERWIN B, FLOOR E, AND MARTIN TF. CAPS (mammalian unc-13) protein localizes to membranes involved in dense-core vesicle exocytosis. *Neuron* 21: 137–145, 1998.
- BI GQ, MORRIS RL, LIAO G, ALDERTON JM, SCHOLEY JM, AND STEINHARDT RA. Kinesin- and myosin-driven steps of vesicle recruitment for Ca^{2+} -regulated exocytosis. *J Cell Biol* 138: 999–1008, 1997.
- BOCK JB, MATERN HT, PEDEN AA, AND SCHELLER RH. A genomic perspective on membrane compartment organization. *Nature* 409: 839–841, 2001.
- BRANDSTATTER JH, FLETCHER EL, GARNER CC, GUNDELFINGER ED, AND WASSLE H. Differential expression of the presynaptic cytomatrix protein bassoon among ribbon synapses in the mammalian retina. *Eur J Neurosci* 11: 3683–3693, 1999.
- BUNT AH. Enzymatic digestion of synaptic ribbons in amphibian retinal photoreceptors. *Brain Res* 25: 571–577, 1971.
- DANTZIG JA, WALKER JW, TRENTHAM DR, AND GOLDMAN YE. Relaxation of muscle fibers with adenosine 5'-[gamma-thio]triphosphate (ATP[gamma S]) and by laser photolysis of caged ATP[gamma S]: evidence for Ca^{2+} -dependent affinity of rapidly detaching zero-force cross-bridges. *Proc Natl Acad Sci USA* 85: 6716–6720, 1988.
- DICK O, HACK I, ALTROCK WD, GARNER CC, GUNDELFINGER ED, AND BRANDSTATTER JH. Localization of the presynaptic cytomatrix protein Piccolo at ribbon and conventional synapses in the rat retina: comparison with Bassoon. *J Comp Neurol* 439: 224–234, 2001.
- GILLIS KD. Techniques for membrane capacitance measurements. In: *Single-Channel Recordings*. New York: Plenum, 1995, p. 155–198.
- GRYNKIEWICZ G, POENIE M, AND TSJEN RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440–3450, 1985.
- HANSON PI, HEUSER JE, AND JAHN R. Neurotransmitter release—four years of SNARE complexes. *Curr Opin Neurobiol* 7: 310–315, 1997a.
- HANSON PI, ROTH R, MORISAKI H, JAHN R, AND HEUSER JE. Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell* 90: 523–535, 1997b.
- HAY JC AND MARTIN TF. Resolution of regulated secretion into sequential MgATP-dependent and calcium-dependent stages mediated by distinct cytosolic proteins. *J Cell Biol* 119: 139–151, 1992.
- HAY JC AND MARTIN TF. Phosphatidylinositol transfer protein required for ATP-dependent priming of $Ca(2+)$ -activated secretion. *Nature* 366: 572–575, 1993.
- HAY JC, FISETTE PL, JENKINS GH, FUKAMI K, TAKENAWA T, ANDERSON RA, AND MARTIN TF. ATP-dependent inositol phosphorylation required for $Ca(2+)$ -activated secretion. *Nature* 374: 173–177, 1995.
- HAY JC AND SCHELLER RH. SNAREs and NSF in targeted membrane fusion. *Curr Opin Cell Biol* 9: 505–512, 1997.
- HEIDELBERGER R. Adenosine triphosphate and the late steps in calcium-dependent exocytosis at a ribbon synapse. *J Gen Physiol* 111: 225–241, 1998.
- HEIDELBERGER R. ATP is required at an early step in compensatory endocytosis in synaptic terminals. *J Neurosci* 21: 6467–6474, 2001a.
- HEIDELBERGER R. Electrophysiological approaches to the study of neuronal exocytosis and synaptic vesicle dynamics. *Rev Physiol Biochem Pharmacol* 143: 1–80, 2001b.
- HEIDELBERGER R AND MATTHEWS G. Calcium influx and calcium current in single synaptic terminals of goldfish retinal bipolar neurons. *J Physiol (Lond)* 447: 235–256, 1992.
- HENRY WR AND MULROY MJ. Afferent synaptic changes in auditory hair cells during noise-induced temporary threshold shift. *Hear Res* 84: 81–90, 1995.

- HOLZ RW, BITTNER MA, PEPPERS SC, SENTER RA, AND EBERHARD DA. MgATP-independent and MgATP-dependent exocytosis. Evidence that MgATP primes adrenal chromaffin cells to undergo exocytosis. *J Biol Chem* 264: 5412–5419, 1989.
- ISSA NP AND HUDSPETH AJ. Clustering of Ca²⁺ channels and Ca²⁺-activated K⁺ channels at fluorescently labeled presynaptic active zones of hair cells. *Proc Natl Acad Sci USA* 91: 7578–7582, 1994.
- JAHN R AND SÜDHOF TC. Membrane fusion and exocytosis. *Annu Rev Biochem* 68: 863–911, 1999.
- KAWASAKI F, MATTIUIZ AM, AND ORDWAY RW. Synaptic physiology and ultrastructure in comatose mutants define an in vivo role for NSF in neurotransmitter release. *J Neurosci* 8: 10241–102419, 1989.
- KLENCHIN VA AND MARTIN TF. Priming in exocytosis: attaining fusion competence after vesicle docking. *Biochimie* 82: 3990407, 2000.
- LENZI D, CRUM J, ELLISMAN MH, AND ROBERTS WM. Depolarization depletes synaptic vesicles in frog saccular hair cells. *Biophys J* 78: 260A, 2000.
- LENZI D, RUNYEON JW, CRUM J, ELLISMAN MH, AND ROBERTS WM. Synaptic vesicle populations in saccular hair cells reconstructed by electron tomography. *J Neurosci* 19: 119–132, 1999.
- LENZI D AND VON GERSDORFF H. Structure suggests function: the case for synaptic ribbons as exocytotic nanomachines. *Bioessays* 23: 831–840, 2001.
- LITTLETON JT, BARNARD RJO, TITUS SA, SLIND J, CHAPMAN ER, AND GANETZKY B. SNARE-complex disassembly by NSF follows synaptic vesicle fusion. *Proc Natl Acad Sci USA* 98: 12233–12238, 2001.
- LOYET KM, KOWALCHYK JA, CHAUDHARY A, CHEN J, PRESTWICH GD, AND MARTIN TFJ. Specific binding of phosphatidylinositol 4,5-bisphosphate to calcium-dependent activator protein for secretion (CAPS), a potential phosphoinositide effector protein for regulated exocytosis. *J Biol Chem* 273: 8337–8343.
- MENNERICK S AND MATTHEWS G. Ultrafast exocytosis elicited by calcium current in synaptic terminals of retinal bipolar neurons. *Neuron* 17: 1241–1249, 1996.
- MOCHIDA S, KOBAYASHI H, MATSUDA Y, YUDA Y, MURAMOTO K, AND NONOMURA Y. Myosin II is involved in transmitter release at synapses formed between rat sympathetic neurons in culture. *Neuron* 13: 1131–1142, 1994.
- MORALES M, COLICOS MA, AND GODA Y. Actin-dependent regulation of neurotransmitter release at central synapses. *Neuron* 27: 539–550, 2000.
- MORGANS CW. Localization of the alpha(1F) calcium channel subunit in the rat retina. *Invest Ophthalmol Vis Sci* 42: 2414–2418, 2001.
- MORGANS CW, BERNTSON A, AND TAYLOR WR. Presynaptic α 1F calcium channels in bipolar cells of the mouse retina. *Soc Neurosci Abstr* 27: 284.14, 2001.
- MURESAN V, LYASS A, AND SCHNAPP BJ. The kinesin motor KIF3A is a component of the presynaptic ribbon in vertebrate photoreceptors. *J Neurosci* 19: 1027–1037, 1999.
- OTTO H, HANSON PI, AND JAHN R. Assembly and disassembly of a ternary complex of synaptobrevin, syntaxin, and SNAP-25 in the membrane of synaptic vesicles. *Proc Natl Acad Sci USA* 94: 6197–6201, 1997.
- PARSONS TD, COORSSSEN JR, HORSTMANN H, AND ALMERS W. Docked granules, the exocytic burst, and the need for ATP hydrolysis in endocrine cells. *Neuron* 15: 1085–1096, 1995.
- PUSCH M AND NEHER E. Rates of diffusional exchange between small cells and a measuring patch pipette. *Pflügers Arch* 411: 204–11, 1988.
- RAVIOLA E AND RAVIOLA G. Structure of the synaptic membranes in the inner plexiform layer of the retina: a freeze-fracture study in monkeys and rabbits. *J Comp Neurol* 209: 233–248, 1982.
- RENDEN R, BERWIN B, DAVIS W, ANN K, CHIH C-T, KREBER R, GANETZKY B, MARTIN TFJ, AND BROADIE K. Drosophila CAPS is an essential gene that regulates dense-core vesicle release and synaptic vesicle fusion. *Neuron* 31: 421–437, 2001.
- ROMBERG L AND VALE RD. Chemomechanical cycle of kinesin differs from that of myosin. *Nature* 361: 168–170, 1993.
- RYAN TA, LI L, CHIN LS, GREENGARD P, AND SMITH SJ. Synaptic vesicle recycling in synapsin I knock-out mice. *J Cell Biol* 134: 1219–1227, 1996.
- SAKABA T, TACHIBANA M, MATSUI K, AND MINAMI N. Two components of transmitter release in retinal bipolar cells: exocytosis and mobilization of synaptic vesicles. *Neurosci Res* 27: 357–370, 1997.
- SCHLAMP CL AND WILLIAMS DS. Myosin V in the retina: localization in the rod photoreceptor synapse. *Exp Eye Res* 63: 613–619, 1996.
- SCHMITZ F, KONIGSTÖRFER A, AND SÜDHOF TC. RIBEYE, a component of synaptic ribbons: a protein's journey through evolution provides insight into synaptic ribbon function. *Neuron* 28: 857–872, 2001.
- SCHNELLE E AND NICOLL RA. Hippocampal synaptic transmission and plasticity are preserved in myosin Va mutant mice. *J Neurophysiol* 85: 1498–1501, 2001.
- SCHWEIZER FE, DRESBACH T, DEBELLO WM, O'CONNOR V, AUGUSTINE GJ, AND BETZ H. Regulation of neurotransmitter release kinetics by NSF. *Science* 279: 1203–1206, 1998.
- SHIMIZU T, FURUSAWA K, OHASHI S, TOYOSHIMA YY, OKUNO M, MALIK F, AND VALE RD. Nucleotide specificity of the enzymatic and motile activities of dynein, kinesin, and heavy meromyosin. *J Cell Biol* 112: 1189–1197, 1991.
- SOKAC AM AND BEMENT WM. Regulation and expression of metazoan unconventional myosins. *Int Rev Cytol* 200: 197–304, 2000.
- TACHIBANA M AND OKADA T. Release of endogenous excitatory amino acids from ON-type bipolar cells isolated from the goldfish retina. *J Neurosci* 11: 2199–2208, 1991.
- TACHIBANA M, OKADA T, ARIMURA T, KOBAYASHI K, AND PICCOLINO M. Dihydropyridine-sensitive calcium current mediates neurotransmitter release from bipolar cells of the goldfish retina. *J Neurosci* 13: 2898–2909, 1993.
- TANDON A, BANNYKH S, KOWALCHYK JA, BANERJEE A, MARTIN TF, AND BALCH WE. Differential regulation of exocytosis by calcium and CAPS in semi-intact synaptosomes. *Neuron* 21: 147–154, 1998.
- TOLAR LA AND PALLANCK L. NSF function in neurotransmitter release involves rearrangement of the SNARE complex downstream of synaptic vesicle docking. *J Neurosci* 18: 10250–10256, 1998.
- UNGERMANN C, SATO K, AND WICKNER W. Defining the functions of trans-SNARE pairs. *Nature* 396: 543–548, 1998.
- VON GERSDORFF H AND MATTHEWS G. Dynamics of synaptic vesicle fusion and membrane retrieval in synaptic terminals. *Nature* 367: 735–739, 1994a.
- VON GERSDORFF H AND MATTHEWS G. Depletion and replenishment of vesicle pools at a ribbon-type synaptic terminal. *J Neurosci* 17: 1919–1927, 1997.
- VON GERSDORFF H, SAKABA T, BERGLUND K, AND TACHIBANA M. Submillisecond kinetics of glutamate release from a sensory synapse. *Neuron* 21: 1177–1188, 1998.
- VON GERSDORFF H, VARDI E, MATTHEWS G, AND STERLING P. Evidence that vesicles on the synaptic ribbon of retinal bipolar neurons can be rapidly released. *Neuron* 16: 1221–1227, 1996.
- XU T, ASHERY U, BURGOYNE RD, AND NEHER E. Early requirement for α -SNAP and NSF in the secretory cascade in chromaffin cells. *EMBO J* 18: 3293–3304, 1999.
- XU T, BINZ T, NIEMANN H, AND NEHER E. Multiple kinetic components of exocytosis distinguished by neurotoxin sensitivity. *Nat Neurosci* 1: 192–200, 1998.
- ZENISEK D AND MATTHEWS G. The role of mitochondria in presynaptic calcium handling at a ribbon synapse. *Neuron* 25: 229–237, 2000.