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Topical Review

Interaction of Secretory Organelles with the Membrane

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Introduction

Neurones and neuroendocrine cells release transmitters and hormones by exocytosis of secretory vesicles or granules. To become available for release, these membrane-delimited organelles move from the cytoplasm to the plasma membrane where they get immobilized. Upon stimulation, secretory organelles at the periphery of the cell fuse with the cell's surface, where their cargo is liberated. After exocytosis, the vesicle's membrane is retrieved, internalized and made available for a new round of vesicular release. The relative amount and kinetics of these ins and outs of vesicles has been modeled, based on plastic changes in the measured rate of release in response to repeated stimuli exhausting the secretory machinery. The identification of the molecular players that orchestrate the regulation of various pre-exocytotic and postfusion steps has only begun, and the sequence of their interactions remains largely unknown. Now, many aspects of the vesicle cycle can be studied in unprecedented detail in a variety of cell types, thanks largely to new tools of molecular analysis, improvements in electrophysiological monitoring and recent advances in optical imaging. A combination of these methods appears promising not only for testing specific hypotheses and for defining constraints of molecular models, but also for bringing studies of individual synaptic vesicle-dynamics into reach (Zenisek et al., 2000).

Various aspects of the vesicle cycle have been reviewed recently. These include the molecules orchestrating the vesicle cycle (Bajjalieh & Scheller, 1995; Südhof, 1995; Hanson et al., 1997; Burger & Schaefer, 1998; Fernandez-Chacon & Südhof, 1999; Bajjalieh, 1999), the role of intracellular free calcium ($[Ca^{2+}]_i$) (Südhof & Rizo, 1996; Zucker, 1996; Neher, 1998; Zucker, 1999), the behavior and fate of the fusion pore (Betz & Angleson, 1998) and different aspects of membrane retrieval (Matthews, 1996; Angleson & Betz, 1997; Betz & Angleson, 1998; Cochilla et al., 1999; Murthy, 1999; Gersdorff & Matthews, 1999). In this review, we focus on (i) recent advances in dissecting intracellular populations of vesicles in different degrees of "readiness" for secretion, and (ii) on the optical tracking of vesicles and granules during their intracellular trafficking.

Vesicle Cycling Sustains High Rates of Transmitter Release

Early on, it was recognized that the release of transmitter into the synaptic cleft involves the secretion of quantal packets (Katz, 1966) and the tightly regulated cycling of membrane-delimited transport organelles to sustain high rates of secretion (Ceccarelli & Hurlbut, 1980). Early work relied on the postsynaptic electrical response as the primary reporter of synaptic vesicle fusion and liberation of neurotransmitter (summarized in Rahamimoff & Fernandez, 1997). As a number of steps are in between exocytosis and the recording of postsynaptic potential, this method has suffered from non-unique interpretation of data. Proof of the vesicular hypothesis and indeed

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much of our knowledge on vesicle cycling (*see* Betz & Angleson, 1998 for a recent overview) has come from the secretion of peptides and hormones in neuroendocrine cells. The tenfold diameter of large dense-core granules compared to synaptic vesicles permitted patch-clamp capacitance (*see* Gillis, 1994) and amperometric measurements (reviewed, e.g., in Chow & von Rüden, 1994) at a single-granule resolution as well as the optical imaging of individual exocytic events (*see* Angleson & Betz, 1997; Murthy, 1999 for review) so that neuroendocrine secretion has become one of the best studied examples in terms of regulatory mechanisms of membrane interactions. Although transmitter release and liberation of hormones, ATP and transmitter differ in some important aspects (Edwards et al., 1996; Kasai, 1999) the basic protein machinery mediating membrane interactions is believed to be very similar in neurones and neuroendocrine cells (Burgoyne & Morgan, 1998; Burgoyne et al., 1996; Burgoyne & Williams, 1997; Artalejo et al., 1998; Ales et al., 1999).

Until recently, with the exception of some invertebrate giant synapses (Katz & Miledi, 1965; Katz, 1969), terminals from retinal bipolar neurones of goldfish (Gersdorff et al., 1996; Heidelberger & Matthews, 1996; Lagano et al., 1996; Matthews, 1996; Job & Lagnado, 1998; Neves & Lagnado, 1999; Zenisek et al., 1999) and the giant brainstem synapse, calyx of Held (Borst et al., 1995; Gersdorff et al., 1997; Weis et al., 1999; Schneggenburger et al., 1999), most synapses have not been very accessible to experimental manipulation, making neuroendocrine cells important 'model nerve terminals.' The recent improvement of the measuring techniques of secretion (Albillos et al., 1997; Ales et al., 1999; Zenisek et al., 1999; Lindau et al., 1999) brings single-vesicle studies in individual nerve terminals within reach and will allow a direct comparison of the exocytosis of vesicles and granules.

Vesicles Mature Through Successive Pools

A well-known trait of stimulated exocytosis is the accumulation or docking of transmitter-containing vesicles beneath the plasma membrane, where they seem to be blocked until fusion is induced by Ca^{2+}-influx through voltage-activated Ca^{2+}-channels. Docked vesicles have been assumed to underlie the rapid response to stimulation which is a characteristic feature of synaptic transmission. Recent experiments have demonstrated in many cell types that only a subpopulation of these "morphologically" docked vesicles or granules can be recruited for immediate release. The transition a from 'resting' to fusion-competent vesicles is one mechanism that regulates the rate of secretion (Augustine & Neher, 1992; Rüden & Neher, 1993; Ryan, Ziv & Smith, 1996) and has been implicated in plastic changes of the transmission efficiency of central synapses (Elmquist & Quastel, 1965; Betz, 1970; Pieribone et al., 1995; Rosenmund & Stevens, 1996; Ryan et al., 1996). Similarly, neuroendocrine cells undergo a secretory depression following intense stimulation (Neher & Zucker, 1993; Thomas et al., 1993; Horrigan & Bookman, 1994; Moser & Neher, 1997), augmentation following elevation of cytoplasmic calcium ($[Ca^{2+}]_i$) to submicromolar levels (Thomas et al., 1993; Bittner & Holz, 1992; Rüden & Neher, 1993), and long-lasting potentiation upon activation of protein kinase A (PKA) (Knight & Baker, 1983; Ämmälä et al., 1994; Vitale et al., 1995; Gillis et al., 1996). Depletion, refill, or overfill of a pool of readily releasable quanta have often been proposed as mechanisms for the observed changes in the rate of secretion. In general, the morphologically and presumably biochemically docked pool will be different from the previous one. While the readily releasable vesicle pool can be measured with standard techniques (*see below*), the dynamics of the vesicle population has been hidden from the experimenter. It would be important to know, which fraction of the docked pool belongs to the readily releasable pool, and the interaction between these two populations. The situation may be even more complicated as empty synaptic vesicles seem to recycle and undergo exocytosis too (Parsons et al., 1999). In the absence of adequate experimental tools the size of the release-ready pool has been difficult to prove directly since only released vesicles show up in standard assays for secretion. To define the role of pool depletion in plastic changes of the rate of release, the size of the pool and the fraction released during a unitary stimulus must be known.

Estimating the Size of the Release-Ready Pool

The size of the release-ready pool was estimated at varied times from cell-capacitance jumps in response to paired membrane depolarizations (Gillis et al., 1996; Gillis & Chow, 1997; Heinemann et al., 1993; Smith et al., 1998; Ashery et al., 1999). Simple linear kinetic models assumed sequentially arranged "immediately" and "readily" releasable granule populations (Heinemann et al., 1993; Rüden & Neher, 1993; Horrigan & Bookman, 1994; Neher & Zucker, 1993; Parsons et al., 1995) and the rate-limiting mobilization of vesicles from a larger "reserve pool" to refill the release-ready pool (Heinemann et al., 1993; Oheim et al., 1999*a*). First-order differential equations describe the trafficking of granules between the release-ready pool and at least one reserve pool in these models and yield satisfactory estimates of the secretory response. The experimentally observed time-course of pool recovery and steady-state size could be predicted from the measured $[Ca^{2+}]_i$ signal assuming Ca^{2+}-dependent supply of granules (Smith et al., 1998; *see* Neher, 1998, for review). In summary, previous bio-

physical models of regulated exocytosis emphasize (i) the presence of a limited pool of release-ready granules in close apposition to the plasma membrane, (ii) have generally assumed the functional homogeneity of this granule population (i.e., albeit having different sizes and locations, one type of granule is assumed). Granules have a common history and fate, an assumption that has recently undergone modification by a number of findings (Thomas-Reetz & DeCamilli, 1994; Kasai et al., 1996; Koenig & Ikeda, 1996; Smith et al., 1998; Parsons et al., 1999). Another stronghold of biophysical models that has come under fire is (iii) the linear kinetic reaction scheme relating the reserve-pool, release-ready pool (RRP) and the pool of secreted granules (Smith et al., 1998; Parsons et al., 1999). Common features of many models are (iv) a dependence on the intracellular free calcium concentration ($[Ca^{2+}]_i$) of the rate-constant for exocytosis with a third- or fourth-power law. (v) a Michaelis-Menten type regulation of the rate of supply of "reserve" granules to the RRP. One of the consequences of this model is the sensitivity of granule fusion to local elevations in $[Ca^{2+}]_i$ ("Ca-microdomains," *see* Neher, 1998). Two recent papers have added a new dimension to this model with the discovery of PKC-dependent and independent $[Ca^{2+}]_i$-regulated supply pathways to the RRP (Smith, 1999) and a switching between different modes of secretion as a function of $[Ca^{2+}]_i$ (Ales et al., 1999).

Among the studied synapses, the giant presynaptic terminals of goldfish retina bipolar neurones (Gersdorff & Matthews, 1999) and the Calyx of Held (Gersdorff et al., 1997; Weis et al., 1999; Schneggenburger et al., 1999; Hori et al., 1999) have been used to estimate the released fraction and total size of the releasable pool. The spherical single synaptic terminals of bipolar neurones are particularly suited for the capacitance-type of analysis (Gillis & Chow, 1997) as are chromaffin cells: capacitance responses from bipolar-cell synaptic terminals saturate for longer depolarizing pulses, that — with the knowledge of the single-vesicle capacitance — have been converted into the number of granules released (Gersdorff & Matthews, 1997). Recently, the question of pool size has been addressed in an intact calyx synapse (Schneggenburger et al., 1999). Excitatory postsynaptic currents were recorded as a measure of presynaptic transmitter release, and Ca^{2+}-influx through voltage-activated Ca^{2+}-channels as well as flash-photolysis caged-Ca^{2+} were used to saturate the Ca^{2+}-evoked transmitter release. These studies have confirmed and extended models previously developed for neuroendocrine secretion to synaptic preparations.

Dissecting Kinetic Intermediates

While compartmental modeling provides a convenient way to explain dynamic changes in the rate of secretion (Neher, 1998; Weis et al., 1999), the molecular basis of 'fusion competence,' and what features characterize the different 'reserve,' 'docked,' 'release-ready' and 'immediately releasable' granule- and vesicle pools are less clear. One attractive in vitro model that has been used to study protein-protein interactions and membrane fusion is the homotypic fusion of sea urchin egg cortical vesicles (CV) (Zimmerberg et al., 1999). Homologues of vesicle-associated membrane protein (VAMP), syntaxin, and SNAP-25 were identified in CV membranes (Tahara et al., 1998). Zimmerberg's group investigated the role of calcium-dependent inactivation in submaximal secretory responses that only release a fraction of the vesicle pool in sea urchin eggs (Blank et al., 1998). They concluded that the cessation of fusion in the continued presence of calcium was not due to calcium-dependent inactivation. Rather, the calcium-sensitivity of individual vesicles within a population of exocytic vesicles was heterogeneous. A calcium concentration above threshold triggered subpopulations of vesicles to fuse, and the size of the recruited vesicle pool was dependent upon the magnitude of the calcium stimulus (Blank et al., 1998). Whereas, several years after the original SNARE hypothesis, there is compelling evidence that docking and membrane fusion are mediated by a tightly regulated sequence of protein-protein interactions (Südhof, 1995; Hanson, Heuser, & Jahn, 1997; Jahn & Südhof, 1994; Rothman & Söllner, 1997; Jahn, 1998; Augustine et al., 1999; Bajjalieh, 1999), little is known on the preceding translocation steps from the cytosol to the granules' docking sites at the plasma membrane. Clostridial neurotoxins have served as molecular tools to dissect different stages of protein assembly (Penner et al., 1986; Bittner & Holz, 1993; Jahn & Niemann, 1994; Glenn & Burgoyne, 1996; Xu et al., 1998), and have revealed important details on the 'late' steps in secretion control. Temperature and intracellular [ATP] change the speed of granule maturation (Bittner & Holz, 1992*a*,*b*; Jankowski et al., 1993; Parsons et al., 1995, 1996; Cole, 1999), but may be too unspecific to be involved in the transition from one particular state to another.

Visualizing Individual Granules

High granule densities near the plasma membrane, and the fluorescence blur due to out-of-focus fluorescence excitation have prevented resolving individual granules in most cell types (*see* however, Angleson et al., 1999). In synaptic preparations, the problem is aggravated as synaptic vesicles are an order of magnitude smaller than neuroendocrine granules. CLSM (Pawley, 1995) and nonlinear fluorescence excitation (Denk et al., 1990) provide excellent optical sectioning and have been used for imaging single-granule dynamics (Burke et al., 1997; Maiti et al., 1997; Levitan, 1998). However, in studies

of regulated exocytosis their benefit has been limited due to inherent drawbacks: confocal detection makes very inefficient use of excitation light (as a rule of thumb, less than 1% are captured) so that long illumination periods introduce a high radiation burden on the cell. Signal levels in multi-photon excitation scanning microscopy are typically low compared to conventional wide-field techniques which is disadvantageous for fast imaging applications and observing multiple sites of release at the same time (Tan et al., 1999; Koester et al., 1999). One alternative in place of confining the readout- or excitation-volume, proposed by Ryan et al. (1997) is to confine the labeling to a few vesicles instead of many (Betz & Angleson, 1997).

Seeing is Believing

The emergence of new optical measurement techniques and the development of vesicle- or membrane-specific stains have been crucial for enabling the measurements of exo- and endocytosis (Smith & Betz, 1996; Betz et al., 1996; Betz & Angleson, 1997; Cochilla et al., 1999; Murthy, 1999; Angleson et al., 1999). Although not really novel techniques, confocal laser scanning microscopy (CLSM) and evanescent-wave (EW) excitation of fluorescence have only recently been applied to the direct observation of individual-granule turnover (Burke et al., 1997; Steyer et al., 1997; Lang et al., 1997; Oheim et al., 1998; Johns et al., 1999). Tracking of cytosolic single-granule positions in neuroendocrine cells (Oheim et al., 1999*a*,*b*; Steyer & Almers, 1999) and fluorescence correlation spectroscopy (FCS) in synaptic nerve endings (Jordan & Klingauf, 2000) provided data on the mobility and modes of motion of secretory organelles at different stages preceding exocytosis. While previous evidence for functional vesicle pools has been indirect and model-dependent (*see* Gillis & Chow, 1997 for a critical discussion), the use of CLSM (Burke et al., 1997; Wacker et al., 1997), EW-fluorescence excitation (Lang et al., 1997; Steyer, Horstmann & Almers, 1997; Oheim et al., 1998), and electron tomography (Lenzi et al., 1999) has complemented earlier EM-data with detailed morphological and functional data.

A Minor Revolution in the Way Individual Granules Can Be Studied

Evanescent-wave imaging (Fig. 1*A*) is an optical sectioning technique that is based on total reflection of light at a dielectric interface (panel *A*1). For cells grown in culture, light incident at an angle exceeding the critical angle is reflected at the glass-water interface. A thin optical near-field is set up in cells grown on the glass substrate. Fluorescently labeled secretory organelles are visualized when they enter a ≈200-nm slab of cytosol beneath the plasma membrane, close to the coverslip on which the cell is grown. Granules (at the limits of optical resolution) and individual (subresolution) vesicles show up as fluorescent spots with an intensity getting brighter the deeper they immerse into the near-field, i.e., the closer they approach the membrane. In a very elegant combination of amperometry, interference-reflection contrast microscopy and evanescent-wave imaging, Steyer et al. (1997) established the method for chromaffin granules (*see* panel *A*2 and Steyer et al., 1997). Further evidence came from the observed granule densities, the stimulation-dependent disappearance of some of them with a kinetics consistent with exocytosis and the supply of new granules to the membrane with a time-constant of ≈6 min (Steyer et al., 1997; Oheim et al., 1998). Using bulk labeling of the intravesicular volume, release is evidenced by the appearance of a cloud of liberated dye molecules into the extracellular space that quickly gets diluted (Steyer et al., 1997; Oheim et al., 1998).

Evanescent-wave imaging visualizes details of individual granule movement in a variety of neuroendocrine cells, among which bovine chromaffin cells (Oheim et al., 1999*a*,*b*; Steyer & Almers, 1999; Loerke et al., 2000), synaptotagmin-1 deficient mouse-chromaffin cells (Loerke et al., 1998), PC-12 cells (Levitan, 1998), and rat pituitary gonadotrophs (T. Fiordelisio, D. Loerke and M. Oheim, *unpublished results*). The same technique has recently been applied to visualize individual synaptic vesicles in nerve endings of hippocampal CA1 neurones in organotypic culture (M. Oheim, *unpublished*), and retinal bipolar nerve terminals (Zenisek et al., 1999).

Whereas imaging with virtually no background (panel *A*2), the use of wide-field detection, and the reduction of photodamage due to the confinement of excitation light constitute the foremost advantages for imaging individual granules (summarized in Oheim & Loerke, 1999), axial positional information is contained in the decay of the EW-intensity with increasing distance from the reflecting interface. The granules' approach to the membrane is particularly well resolved when small penetration depths of the EW-field decay magnify the relative fluorescence change. Additionally, quantitative estimates of membrane orientation (Sund et al., 1999; Mertz, 2000), intragranular dye concentration (Loerke et al., 2000), and the topography of cell adhesion (Ölveczky et al., 1997; Oheim & Stühmer, 2000) are obtained from recent variants of evanescent-wave imaging (*see* Oheim, 2000 for overview).

Look How They Fuse: Transport Docking and Release of Secretory Granules

Recent advances in optical imaging have shed light on the ins and outs of granules to and from a 200-nm slice

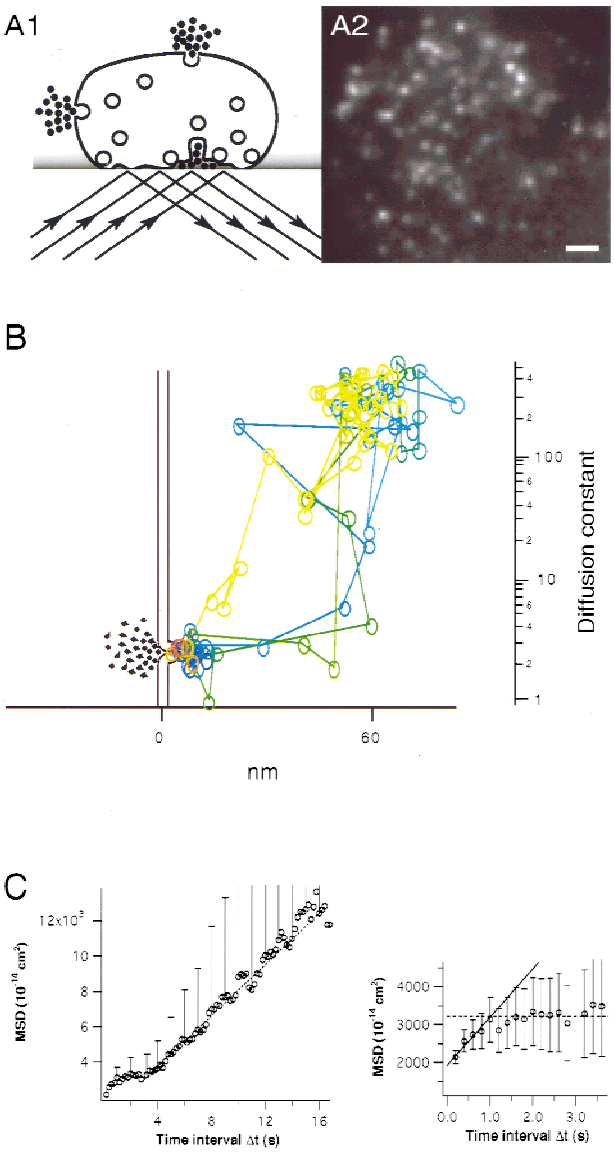

Fig. 1. Fluorescently labeled granules and vesicles can be observed intracellularly, prior to exocytosis, using evanescent-wave excitation. (*A*1) schematic drawing of a neurosecretory cell grown in culture on a glass coverslip. A laser beam is directed at the dielectric interface between the glass and the aqueous solution so that it undergoes total reflection. In a thin layer above the interface there is light, as an evanescent near-field is set up that rapidly decays with increasing distance (typically within λ/5 to λ/2, where λ denotes the wavelength of light) from the interface. Only fluorophores present within this tiny excitation volume light up while the bulk of the cytoplasm and the solution topping the cell remain dark. (*A*2) Evanescent-wave excited image of a bovine adrenal chromaffin cell. Secretory granules were labeled with acridine orange and show up as individual pinpoints (© Springer-Verlag, *Eur. Biophys. J.* (2000)). Particle tracking and the quantitative interpretation of fluorescence intensity changes were used to derive the three-dimensional trajectory of the granules. (*B*) Parametric plot of granule mobility *vs.* distance to the plasma membrane (shaded line). Time is encoded in pseudo-color from $t = 0$ (blue) until the fusion of the granule with the membrane (red). The granule is seen to progress from a relatively highly mobile state at ≈60-nm distance from the membrane to a virtually immobile state at the membrane. The granule's mobility is expressed as the short-range free diffusion coefficient (in 10^{-12} cm^{2}sec^{-1}) and is several thousand times slower compared with value for a granule-sized sphere in a liquid of the cytoplasm's viscosity. Membrane fusion is evidenced as the brief appearance of a cloud of released dye molecules. (*C*) For individual granules, the mean-squared displacement (MSD) was calculated as a function of the observation time. Whereas a linear curve indicates free diffusion, negative curvature is interpreted as diffusion through a mesh. The size of the "cage" can be estimated from the asymptote of the MSD *vs.* Δt plot for $\Delta t \rightarrow \infty$ (dashed line). Cage sizes are typically only slightly larger than the granule.

near the plasma membrane. The detailed analysis of individual-granule motion is one unique advantage of optical imaging (*see* the review by Murthy, 1999). Optical imaging comes to its real power when applied simultaneously to large populations of granules, using automated tracking algorithms (Gosh & Webb, 1994) and the statistical analysis of many granule trajectories (Steyer & Almers, 1999; Oheim & Stühmer, 2000). With its confined volume of fluorescence excitation EW-imaging permits the acquisition of long image series without measurable photodamage (Steyer & Almers, 1999; Oheim & Stühmer, 2000; Oheim, 2000). Optical imaging has not only permitted the study of granular dynamics beneath the plasma membrane (Steyer et al., 1997; Lang et al., 1997; Oheim et al., 1998), but provided direct evidence for functionally different granule populations, based on mobility data and the distance of the granule to the membrane (Oheim et al., 1999*a*,*b*; Steyer & Almers, 1999). Unlike capacitance or amperometric measurements, optical imaging has provided estimates, e.g., of the distance traveled, the dwell time of secretory organelles in different compartments (Levitan, 1998; Hirschberg et al., 1998; Murthy, 1999), the mode of motion (Steyer & Almers, 1999; Oheim & Stühmer, 2000), its direction and speed (Oheim et al., 1999*a*,*b*), but also parameters like the intragranular dye content (Angleson et al., 1999; Loerke et al., 1999), pH (Miesenböck & Rothman, 1997; Miesenböck et al., 1998), swelling of the granule (Loerke et al., 2000; Loerke et al., 1999), or the presence of particular molecules on granules and vesicles (Lang et al., 1997; Levitan, 1998; Lang et al., 2000). Measuring one or more of these parameters over time, functionally different states of granules *en route* for exocytosis have been identified without

prior assumption of a particular kinetic scheme or model (Oheim et al., 1999*a*,*b*; Oheim & Stühmer, 2000).

Immobilization is a Regulatory Process Preparatory to Membrane Fusion

On time-resolved image stacks, granules arrived in the observational volume, moved slowly on random paths while approaching the plasma membrane (evidenced by a concomitant increase in fluorescence intensity), and got immobilized at nor near the plasma membrane, where they stayed virtually immobile in the absence of stimulation. Up to 30% of the granules resumed their wandering later on, and docked anew (Steyer et al., 1997; Oheim et al., 1998; Oheim et al., 1999; Steyer & Almers, 1999). The trajectory of a single vesicle from the time of its appearance to its disappearance, due to exocytosis, is shown in Fig. 1, panel *B*1, and as a parametric plot of its mobility *vs.* distance to the plasma membrane, panel *B*2. The vesicle moved from a distant location at relatively high mobility to a near-membrane state in which the granule is virtually immobile.

In the absence of stimulation, a dynamic equilibrium keeps the sizes of the immobilized and mobile constant; while the sizes of the two visible granule populations remained constant in the absense of stimulation, vesicles occasionally changed from the brighter, less mobile state to the dimmer, highly mobile state, and *vice versa* (Oheim et al., 1999*b*; Steyer & Almers, 1999). In addition, new vesicles spontaneously appeared in the near-membrane region or visible vesicles disappeared at equal rates, indicating a continuous exchange between vesicles that are visible and others in an invisible reserve pool — located deeper in the cell, beyond the reach of the evanescent field (Oheim et al., 1999*a*,*b*). The average swell-time of a granule in the mobile state was 3.5 sec and about 40 sec in the docked state (Oheim et al., 1999*a*).

Membrane Depolarization Shifts the Dynamic Equilibrium of Vesicle Cycling Towards Release

While no spontaneous fusion events were seen in the absence of stimulation, cells started to secrete vigorously after membrane depolarization (Steyer et al., 1997; Oheim et al., 1998). Fluorescent granules disappear as they lose their dye due to exocytosis (rather than re-ascending into the cytoplasm), and this depletes the plasma membrane of docked granules. The increase of release — as monitored by capacitance and amperometric techniques (Heinemann et al., 1993; Heinemann et al., 1994; Smith et al., 1998; Oheim et al., 1999; Smith, 1999) and — albeit less steeply — the concomitant increase in granule supply — assessed indirectly by double-pulse stimulation and monitored by evanescent-wave imaging — depends on the extent and time-course of the $[Ca^{2+}]_i$-elevation. In contrast, the rate-constant of de-docking does not seem to be affected by stimulation. At penetration depths <80 nm of the EW-field, sustained or repetitive stimulation leads to a complete loss of fluorescence from the footprint region of the cell. New granules appear where previously none were visible as granules move from the cytosol toward the plasma membrane. In accordance with earlier models, granules exclusively fused from the immobilized state, confirming a well-defined sequence of events *en route* for exocytosis. In spite of the loss of vesicles after triggering exocytosis, during the initial 15 seconds of maintained membrane depolarization the total number of visible granules remained almost constant. This was due to the recruitment of new vesicles from the reserve pool, located at deeper cytosolic regions and out-of-reach of the EW near-field. Although the rate of recruitment of new granules was enhanced during episodes of exocytosis, the reverse rate-constants (de-docking and leaving the observational volume back into the cytoplasm) remained constant, indicating that these transitions probably depend on the decay or destabilization of a binding complex rather than a Ca-modulated transport process.

Local Cycling and Global Recycling: Regulation of Fast Release

In summary, optical studies of the steps preceding membrane fusion have largely confirmed previous models that were based on more indirect evidence but have highlighted, e.g., the dynamic equilibrium between immobilized and mobile granules in immediate proximity of the plasma membrane. Although reversible docking and maturation steps have been suggested in kinetic schemes (Gillis & Chow, 1997; Heinemann et al., 1993), previous evidence for de-docking has been indirect (Xu et al., 1998). The direct observation of vesicles regaining mobility after immobilization at the plasma membrane corroborates these models and provides time-constants and reaction rates that help to define constraints on the involved protein reactions. The identification of a subgroup of release-ready granules within the "morphologically docked" granule population has been an issue of debate (Parsons et al., 1995; Plattner et al., 1997), and no previous techniques could measure dynamic aspects of granule-trafficking between different functional states. At present, the fluorescence data from neuroendocrine (Oheim et al., 1999*a*,*b*; Steyer & Almers, 1999) and (Burke et al., 1997; Johns et al., 1999) has not converged into a conclusive interpretation of what features unambiguously identify the readiness for release. The observations of Steyer & Almers (1999) and the population

analysis of our own laboratory lead to the conclusion that at least the (morphological) docking reaction is reversible but that granules have to mature through a sequence of states that — if not distinguished by imaging techniques — seem to involve the formation of an immobilized release-ready state as a prelude to membrane fusion. Membrane depolarization of several 100 msec or trains of shorter depolarizations lead to conditions in which the immobilized granule fuses in less than 1 sec allowing the cell to sustain high rates of secretion (Oheim et al., 1999; Oheim & Stühmer, 2000) before exhaustion of a near-membrane pool and slower recovery. Although partial release events have been a relatively rare observation in optical studies, a number of recent studies support the conclusion that the kiss-and-run mechanism (Betz & Angleson, 1998; Artalejo et al., 1998; Ales et al., 1999), possibly combined with local refilling mechanisms may be present in endocrine cells in addition to the "classical" and slow recycling with a time-course of ≈6 min observed with EW-microscopy (Steyer et al., 1997; Oheim et al., 1998). It may well be that partial release events have been masked within what can be resolved with the presently used fluorophores and the attainable combination of time-resolution and dynamic width of the fluorescence signal.

The Future Must be More Colourful and More Specific

The combination of high-resolution imaging like EW-field excitation or fluorescence-resonance energy transfer (FRET)-microscopy with multiple genetically encoded fluorophores in live cells is the obvious next step in studies of regulated secretion. Fluorescent markers like green fluorescent protein (GFP), and its more recent optically enhanced variants, have already been used to label secretory organelles (Burke et al., 1997; Hirschberg et al., 1998; Miesenböck et al., 1998; Wacker et al., 1997; Kaether et al., 1997; Lippincott-Schwartz et al., 1997; Gaidarov et al., 1999), but can be used more specifically when targeted to proteins involved in regulating the vesicle-cycle. The combined use of GFP and its blue variant BFP (Cubitt et al., 1995; Ellenberg et al., 1999), or the more recent yellow and cyan YFP/CFP FRET-pair (Fan et al., 1999) offers the exciting possibility to study vesicle-membrane and protein-protein interactions on a molecular length-scale in live cells (Pollok & Heim et al., 1999; Ha et al., 1996; Weiss, 1999). The recent demonstration of the efficient transfection of chromaffin cells using the Semliki forest virus (Ashery et al., 1999; Duncan et al., 1999; Larsson et al., 1999) paved the way towards a use of these molecular torch-lights in cells that permit optical single-granule tracking. More directly than the use of genetic deletion mutants, e.g., the synaptotagmin-1 knockout (Südhof & Rizo, 1996; Loerke et al., 1998; Geppert et al., 1994; Lund et al., 1997), the fluorescent labeling and observation of specific vesicle- or membrane-associated proteins involved in secretion control allows the clarification of their role during the exo- and endocytic cycle and seems particularly promising to obtain insight into the sequence of events that underlie the maturation of secretory organelles. The main interest in these optical will be threefold, to study (i) molecular assembly of the protein scaffold involved in vesicle docking and catalyzing membrane fusion (Lin & Scheller, 1997; Sutton et al., 1998), and (ii) to investigate morphological integrity of the vesicle after fusion and during endocytosis (Murthy & Stevens, 1998). Equally, (iii) other subgranular aspects of release like postfusion control of secretion and partial release (Betz & Angleson, 1998; Artalejo et al., 1998; Ales, Tabares et al., 1999), *see above* and the commentary in Fesce & Meldolesi (1999), have only begun to be studied.

Further advancement in the field will come from the use of optical studies in combination with other techniques, like Ca^{2+}-measurements, membrane-capacitance techniques, or the introduction of site-specific probes. In many cases, the support for the involvement of specific proteins at certain stages of the vesicle cycle has been indirect i.e., from measurements of the secretory response, or the accumlation of docked granules in electron micrographs. Now, the labeling of specific secretory proteins, introduction of antibodies against proteins, genetic deletion of vesicle and membrane proteins, and modifications of the cytoskeleton (*see below*) can be used to directly probe interactions of the vesicle with the membrane or other cellular structures. Likewise, the combined use of optical markers for vesicle position, acidification, or membrane fusion with fluorescent Ca^{2+}-indicator dyes promises a more direct insight into the Ca^{2+}-regulation of the secretory apparatus. Finally, and somewhat surprisingly, the more recent work on individual-granule and -vesicle tracking has focused on prefusion migration rather than the endocytic limb of the vesicle cycle (*see* however (Merrifield et al., 1999)). Endocytic uptake of membrane-resident amphiphilic dye (e.g., fm1-43 or fm4-64) (Angleson & Betz, 1997; Cochilla et al., 1999) together with high-resolution imaging will advance our knowledge on how individual vesicles (Zenisek et al., 1999) and chromaffin granules (*work currently underway in our laboratory*) are taken up after release and are recycled for another round of exo- and endocytosis.

Spatial and Motional Aspects of Vesicle-Membrane Interactions

Relatively little is known about which molecular motors direct vesicles and granules to the membrane, what

makes docking and fusion sites different from other membrane areas devoid of secretory organelles, and what is the spatial architecture of the near-membrane region. Earlier work on the spatial aspects of secretion control has used indirect evidence from fixed specimens using electron microscopy (Parsons et al., 1995; Steyer et al., 1997; Plattner et al., 1997; Reist et al., 1998) or electron tomography (Lenzi et al., 1999), and the spatial distribution of elevated near-membrane intracellular $[Ca^{2+}]_i$ near patches of clustered Ca^{2+}-channels (Klingauf & Neher, 1997; Naraghi & Neher, 1997; Tucker & Tettiplace, 1995; Llinas et al., 1995; Bertram et al., 1999; *see* Neher, 1998 for review). More direct measurements have been obtained when miniaturized carbon-fiber electrodes (Robinson et al., 1995, 1996) were used to probe secretion at different locations at the cell surface. A detailed tracking of the intracellular movement of individual fluorescently labeled granules and the spatial distribution and repetitive use of sites of release have become possible only very recently. To really answer the question what makes 'hot spots' of exocytotic activity different from other sites on the membrane, these optical techniques must be combined with intracellular Ca^{2+}-imaging or biochemical approaches.

Superimposed Modes of Motion

A major result from granule-tracking studies is that the absolute mobility of chromaffin granules is surprisingly low with distinctive clusters indicative of different mobility populations (Fig. 1*B*). On a plot of the mean-squared displacement (MSD, an indicator of which distance a granule traveled on average in a given time-interval) *vs.* the time of observation different distinctive features of granule-motion can be recognized (Fig. 1*C*). The slope of the MSD is generally seen to decrease with time, revealing a deviation of free diffusion and indicative of granule diffusion through a mesh of obstacles (Faucheux & Libchaber, 1994), or the superimposed diffusion of the granule attached to a slowly drifting larger structure (Saxton, 1993). Although the high-frequency end of granular or vesicular motion is capped at the acquisition frame rate of the CCD detectors, and the recognition of slow processes is limited by the finite observation time, two regimes have been distinguished. For very short observation times; the slope of the MSD gives the free diffusion coefficient of the individual granule, which is in the order of $\sim 10^{-2}$ $\mu m^2 sec^{-1}$ for mobile granules located 200 nm beneath the membrane, and $\approx 10^{-4}$ $\mu m^2 sec^{-1}$ for granules docked beneath the plasma membrane. These values are about 10^5 times less than the diffusion coefficient of an equal-sized sphere in a homogeneous medium with a viscosity similar to that of the cytosol. Following to the high-frequency short-term mobility, the MSD *vs.* time plots, one often reveals a slower mobility component, which saturates for longer observation times, or converges into a constant slope (dashed), representing a diffusion coefficient of $\approx 10^{-2}$ $\mu m^2 sec^{-1}$ (*see* figure legend for details). These findings have been interpreted as the free diffusion of the granule within a very limited volume, only slightly bigger than the granule itself, and the simultaneous and slower diffusion of this cage in the cytoplasm (Steyer & Almers, 1999). An equal but alternative interpretation suggested by Almers and coworkers is that of a granule kept on a leash and the slow drift of the binding site. Although the average mobility of near-membrane granules beneath the plasma membrane is low, granules can achieve surprisingly high instantaneous velocities of several hundred nanometers per second (Oheim et al., 1999*b*; Oheim & Stühmer, 2000).

Interactions with the Cytoskeleton

Despite an ever-increasing number of protein-protein interactions being identified at various stages of the exo- and endocytic cycle (Weber et al., 1998; Chen et al., 1999; Hardi et al., 1999; Hilfiker et al., 1999), the morphological and kinetic properties of this membrane-transport system are less understood. It is not clear, for example, how secretory organelles translocate through the cytoplasm, how long they reside in different intermediate states, and what directs their movement to specific docking sites at the plasma membrane. Dynamic changes in the polymerization of cortical actin have been proposed to propel vesicles to their fusion sites and back into the cytoplasm (Cooper, 1995; Kibble, Barnard & Burgoyne, 1996; Wacker et al., 1997; Steyer & Almers, 1999). Whereas the Ca^{2+}-dependence of the rate and the amount of membrane addition, release and re-uptake have been studied using a variety of electrophysiological, electrochemical and optical approaches (*see* Angleson & Betz, 1997; Gillis & Chow, 1997; Rahamimoff & Fernandez, 1997; Neher, 1998; Murthy, 1999 for review) the involvement of stimulation- and most likely Ca^{2+}-dependent changes of the near-membrane cytoskeleton have remained inaccessible until recently. An involvement of Ca^{2+} in an early, prefusion step has been been known for several years (Rüden & Neher, 1993; Heinemann et al., 1994; Smith et al., 1998; Oheim et al., 1999; Smith, 1999), but the Ca^{2+}-dependent reaction, and the molecular Ca^{2+}-sensor remain to be elucidated. Recently, using the fluorescent tracer fm1-43, T. Ryan has demonstrated that in hippocampal synaptic terminals during action potential firing inhibitors of the myosin light chain kinase reduce the size of the recycling vesicle pool without a significant change in the kinetics of vesicle turnover. Additionally, the mobilization of a re-

serve pool presumably distant from the membrane is impaired whereas the readily-releasable vesicles seem unaffected by the block of the myosin transport system (Ryan et al., 1999).

Tugging Granules Through the Actin Cortex

At the ultrastructural level, at a pre-exocytotic stage, chromaffin granules are found in juxtaposition to the plasma membrane and separated from it by an electron-dense space (Plattner et al., 1997; Aunis et al., 1979; Nakata et al., 1992). At this stage, chromaffin granules are connected to the plasma membrane by filamentous structures, whereas, after stimulation of exocytosis, the sites of granule fusion are devoid of such connecting structures. Based on these data, a cortical-actin barrier has been proposed to regulate access to sites at the plasma membrane (Cheek & Burgoyne, 1986; Burgoyne & Cheek, 1987). Its reorganization may involve changes in actin-filament crosslinking, and interactions with the granule and the plasma membrane that directly regulate the size of the readily releasable pool (Vitale et al., 1995). As granules entangled in the actin cortex do not show up in standard assays for secretion, it is unclear whether cortical actin indeed acts as a physical barrier to prevent granule docking, or if its role is more a regulatory one, e.g., by transiently depolymerizing during exocytosis (Burgoyne & Cheek, 1987; Vitale et al., 1991; Trifaró & Vitale, 1993). In a recent paper (Oheim & Stühmer, 2000), we analyzed granule trajectories from the time of the granule entering the evanescent field to its fusion with the plasma membrane during the application of compounds modifying the cytoskeletal architecture. We altered the viscosity of filamentous (F-) actin by application of latrunculin and jasplakinolide to assess an involvement of the cytoskeleton in granule mobility and regulating the rate of secretion (*See also* Lang et al., 2000).

The involvement of actin bundles — so-called stress fibbers — in mediating focal adhesion has been known for long (Fowler & Pollard, 1982; Fowler & Vale, 1996). Less than 2 min incubation of chromaffin cells with latrunculins caused a decrease in the mobility of mobile granules to ~50% of its control value in untreated cells, while leaving the immobilized and presumably docked granules unaffected. The onset of the secretory response upon membrane depolarization was even slightly enhanced. These observations suggest that F-actin may provide tracks for granule movement rather than act as a mesh hindering a stochastic granule transport. Conversely, stabilizing the actin cortex by administration of jasplakinolide did result in a near-total loss of granule movement. This observation is consistent with the requirement of a space-dependent actin reorganization to drive granule movement, to actively push or direct the granule to its docking site at the plasma membrane. We conclude that peripheral actin restricts granule movement on the one hand, but dynamic changes of actin polymerization on the other hand are required to support granular motility: stimulation-dependent changes in actin viscosity may drive granule movement in the near-membrane region.

Conclusion

Despite considerable progress in the understanding of the cellular elements of secretion control (Neher, 1998) it has remained unclear, whether granules diffuse through the cytoplasm and are captured near the release site or an active transport mechanism directs granules to these sites. Equally, it is unknown why some sites at the membrane are distinct from others (Schroeder et al., 1994; Oheim et al., 1999), and how granules are targeted to them. The observation of constrained granule mobility (Steyer et al., 1997; Oheim et al., 1999*a*,*b*; Steyer & Almers, 1999; Oheim & Stühmer, 2000) has not yet converged into a molecular model of why granule docking and fusion sites are so close together. It is unknown, how long granules reside at the different pre-exocytotic stages, why only docked granules can acquire fusion competence, and what is the significance of reversible 'docking' reaction. Optical and electrochemical detection have emphasized different phases during release from an individual granule. It has remained unclear what is limiting the time course of release at the individual-granule level. Interactions of its core with the local environment indicate a postfusion-regulation of release. It has remained controversial whether vesicles retain their identity after membrane fusion. Does endocytosis take up the same membrane patch that has been added? Elevation of intracellular free [Ca^{2+}] initialises release and triggers multiple mechanisms of membrane uptake. Cells seem to can switch between different recycling mechanisms.

Among the studies that have provided insight into the molecular machinery for the formation, targeting, docking and fusion of secretory organelles, genetic and biochemical approaches have taken a prominent place (Scheller, 1995; Südhof, 1995; Rothman, 1996; Fasshauer et al., 1998*a*). The combined use of the recent biophysical techniques offering single-vesicle resolution and specific biochemical modifications in the protein machinery involved in vesicular transport, and interactions of the vesicle with its target membrane promises to be a powerful approach to address open questions in the field of secretion.

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References

Albillos, A., et al. 1997. *Nature* **389:**509–512

Ales, E., et al. 1999. *Nature Cell Biol.* **1:**40–44

Ämmälä, C., et al. 1994. *Proc. Natl. Acad. Sci. USA* **91:**4343–4347

Angleson, J.K., Betz, W.J. 1997. *Trends Neurosci.* **20:**281–287

Angleson, J.K., et al. 1999. *Nature Neurosci.* **2:**440–445

Artalejo, C.R., Elhamdani, A., Palfry, H.C. 1998. *Current Biol.* **8:**R62–R65

Ashery, U., et al. 1999. *Eur. J. Cell Biol.* **78:**525–532

Aunis, D., Hesketh, J.E., Devilliers, G. 1979. *Cell Tiss. Res.* **197:**433–441

Augustine, G.J., Neher, E. 1992. *J. Physiol.* **450:**247–271

Augustine, G.J., et al. 1999. *J. Physiol.* **520:**33–41

Bajjalieh, S.M., Scheller, R.H. 1995. *J. Biol. Chem.* **270:**1971–1974

Bajjalieh, S.M. 1999. *Curr. Op. Neurobiol.* **9:**321–328

Bertram, R., Smith, G.D., Sherman, A. 1999. *Biophys. J.* **76:**735–750

Betz, W.J. 1970. *J. Physiol.* **206:**629

Betz, W.J., Mao, F., Smith, C.B. 1996. *Curr. Opin. Neurobiol.* **6:**365–371

Betz, W.J., Angleson, J.K. 1997. *Nature* **388:**423–424

Betz, W.J., Angleson, J.K. 1998. *Annu. Rev. Physiol.* **60:**347–363

Bittner, M.A., Holz, R.W. 1992*a*. *J. Biol. Chem.* **267:**16219–16225

Bittner, M.A., Holz, R.W. 1992*b*. *J. Biol. Chem.* **267:**16226–16229

Bittner, M.A., Holz, R.W. 1993. *Cell. Mol. Neurobiol.* **13:**649–664

Blank, P.S., et al. 1998. *J. Gen. Physiol.* **112:**559–567

Borst, J.G.G., Helmchen, F., Sakmann, B. 1995. *J. Physiol.* **489:**825–840

Burger, M.M., Schaefer, T. 1998. *J. Cell. Biochem. Suppl.* **30/31:**103–110

Burgoyne, R.D., Cheek, T.R. 1987. *Biosci. Rep.* **7:**281–288

Burgoyne, R.D., Morgan, A. 1998. *Cell Calcium* **24:**367–376

Burgoyne, R.D., et al. 1996. SNAPs & SNAREs in exocytosis in chromaffin cells. *In:* Molecular Mechanisms of Neurotransmitter Release. p. 653–658

Burgoyne, R.D., Williams, G. 1997. FEBS Lett. **414:**349–352

Burke, N.V., et al. 1997. *Neuron* **19:**1095–1102

Ceccarelli, B., Hurlbut, W.P. 1980. *Physiol. Rev.* **60:**396–441

Cheek, T.R., Burgoyne, R.D. 1986. *FEBS Lett.* **207:**110–114

Chen, Y.A., et al. 1999. *Cell* **97:**165–174

Chow, R.H., von Rüden, L. 1994. Electrochemical detection of secretion from single cells. *In:* Single-Channel Recording. B. Sakmann & E. Neher, Editors. Plenum, NY

Cochilla, A., Angleson, J.K., Betz, W.J. 1999. *Annu. Rev. Neurosci.* **22:**1–10

Cooper, J.A., M.T.J. 1995. *Curr. Op. Cell Biol.* **7:**1–144

Cubitt, A.B., et al. 1995. *TIBS* **20:**448–455

Denk, W., Strickler, J.H., Webb, W.W. 1990. *Science* **248:**73–76

Duncan, R.R., et al. 1999. *Biochem. J.* **342**(3):497–501

Edwards, M.P., et al. 1996. *Investigative Ophthalmology & Visual Science* **37:**1438–1438

Ellenberg, J., Lippincott-Schwartz, Presley, J.F. 1999. *Trends Cell Biol.* **9:**52–56

Elmquist, D., Quastel, D.M.J. 1965. *J. Physiol.* **178:**505–529

Fan, G.Y., et al. 1999. *Biophys. J.* **76:**2412–2420

Fasshauer, D., et al. 1998*a*. *Biochemistry* **37:**10345–10353

Fasshauer, D., et al. 1998*b*. *Proc. Acad. Sci. USA* **95:**15781–15786

Faucheux, L.P., Libchaber, A.J. 1994. *Phys. Rev. E* **49:**5158–5163

Fernandez-Chacon, R., Südhof, T.C. 1999. *Annu. Rev. Physiol.* **61:**753–776

Fesce, R., Meldolesi, J. 1999. *Nature Cell Biol.* **1:**E3–E4

Fowler, V.M., Pollard, H.B. 1982. *Nature* **295:**336–339

Fowler, V.M., Vale, R. 1996. *Curr. Op. Cell Biol.* **8:**1–3

Gaidarov, I., et al. 1999. *Nature Cell Biol.* **1:**1–7

Geppert, M., et al. 1994. *Cell* **79:**717–727

Gersdorff, H.v., Vardi, E., Matthews, G. 1996. *Neuron* **16:**1221–1227

Gersdorff, H.v., Matthews, G.G. 1997. *J. Neurosci.* **17:**1919–1927

Gersdorff, H.v., et al. 1997. *J. Neurosci.* **17:**8137–8146

Gersdorff, H.v., Matthews, G. 1999. *Annu. Rev. Physiol.* **61:**725–752

Gillis, K.D. 1994. Techniques for Membrane Capacitance Measurements. *In:* Single Channel Recording, B. Sakmann & E. Neher, Editors. Plenum, NY

Gillis, K.D., Mößner, R., Neher, E. 1996. *Neuron* **16:**1209–1220

Gillis, K.D., Chow, R.H. 1997. *Cell Dev. Biol.* **8:**133–140

Glenn, D.E., Burgoyne, R.D. 1996. *FEBS Lett.* **386:**137–140

Gosh, R.N., Webb, W.W. 1994. *Biophys. J.* **66:**1301–1318

Ha, T., et al. 1996. *Proc. Natl. Acad. Sci. USA* **93:**6264–6268

Hanson, P.I., Heuser, J.E., Jahn, R. 1997. *Curr. Op. Neurobiol.* **7:**310–315

Hardi, J.M., et al. 1999. *Nature Neurosci.* **2:**119–124

Heidelberger, R., Matthews, G. 1996. *Biophys. J.* **70:**SU310–U310

Heinemann, C., et al. 1993. *Pfluegers Arch.* **424:**105–112

Heinemann, C., et al. 1994. *Biophys. J.* **67:**2546–2557

Hilfiker, S., et al. 1999. *Nature Neurosci.* **2:**104–106

Hirschberg, K., et al. 1998. *J. Cell Biol.* **143:**1485–1503

Hori, T., Takai, Y., Takahashi, T. 1999. *J. Neurosci.* **19:**7262–7267

Horrigan, F.T., Bookman, R.J. 1994. *Neuron* **13:**1119–1129

Jahn, R., Südhof, T.C. 1994. *Annu. Rev. Neurosci.* **17:**219–246

Jahn, R., Niemann, H. 1994. *Ann. NY Acad. Sci.* **733:**245–255

Jahn, R. 1998. *Curr. Biol.* **8:**R856–R858

Jankowski, J.A., et al. 1993. *J. Biol. Chem.* **268:**14694–14700

Job, C., Lagnado, L. 1998. *J. Cell Biol.* **143:**1661–1672

Johns, L.M., et al. 1999. *Biophys. J.* **76:***A*70 (*Abstr.*)

Jordan, R., Klingauf, J. 2000. *Biophys. J.* **78**(2):1539

Kaether, C., et al. 1997. *Eur. J. Cell Biol.* **74:**133–142

Kasai, H., et al. 1996. *J. Physiol.* **494:**53–65

Kasai, H. 1999. *TINS* **22:**88–93

Katz, B., Miledi, R. 1965. *Proc. R. Soc. Lond.* **161:**483–495

Katz, B. 1966. Nerve, Muscle, and Synapse. pp. 129–141. McGraw-Hill, New York

Katz, B. 1969. The Release of Neural Transmitter Substances. Liverpool, England

Kibble, A.V., Barnard, R.J.O., Burgoyne, R.D. 1996. *J. Cell Sci.* **109:**2417–2422

Klingauf, J., Neher, E. 1997. *Biophys. J.* **72:**674–690

Koenig, J.H., Ikeda, K. 1996. *J. Cell Biol.* **135:**797–808

Koester, H.J., et al. 1999. *Biophys. J.* **77:**2226–2236

Knight, D.E., Baker, P.F. 1983. *FEBS Lett.* **160:**98–100

Lagano, L., Gomis, A., Job, C. 1996. *Neuron* **17:**957–967

Lang, T., et al. 1997. *Neuron* **18:**857–863

Lang, T., et al. 2000. *Biophys. J.* **78:**2863–2877

Larsson, B.O., et al. 1999. *Eur. J. Neurosci.* **11:**1981–1987

Lenzi, D., et al. 1999. *J. Neurosci.* **19:**119–132

Levitan, E.S. 1998. *Methods* (*Duluth*) **16:**182–187

Lin, R.C., Scheller, R.H. 1997. *Neuron* **19:**1087–1094

Lindau, M., et al. 1999. *Biophys. J.* **76:***A*68 (*Abstr.*)

Lippincott-Schwartz, J., Smith, C.L. 1997. *Curr. Biol.* **7:**631–639

Lippincott-Schwartz, J., Cole, N., Presley, J. 1999. *Trends Cell Biol.* **8:**16–20

Llinas, R., Sugimori, M., Silver, R.B. 1995. *Neuropharmacology* **34:**1443–1451

Loerke, D., et al. 2000. *J. Biomed. Opt.* **5:**23–30

Loerke, D., Oheim, M., Stühmer, W. 1999. *Biophys. J.* **76:***A*69 (*Abstr.*)

Loerke, D., et al. 1998. *Eur. J. Neurosci.* **10:**217

Lund, P.E., et al. 1997. Synaptotagmin 1 is involved in the regulation of Ca^{2+} induced secretion from adrenal chromaffin cells. *In:* From Membrane to Mind. Göttingen: Georg Thieme Stuttgart, New York

Maiti, S., et al. 1997. *Science* **275:**530–532

Matthews, G. 1996. *Curr. Op. Neurobiol.* **6:**358–364
Matthews, G. 1996. *Biophys. J.* **70:** UPM2 (*Abstr.*)
Merrifield, C.J., et al. 1999. *Nature Cell Biol.* **1:**72–74
Mertz, J. 2000. *J. Soc. Opt. Am. B* **17(11):**1706–1713
Miesenböck, G., Rothman, J.E. 1997. *Proc. Natl. Acad. Sci. USA* **94:**3402–3407
Miesenböck, G., DeAngelis, D.A., Rothman, J.E. 1998. *Nature* **394:**192–195
Moser, T., Neher, E. 1997. *J. Neurosci.* **17:**2314–2323
Murthy, V.N., Stevens, C.F. 1998. *Nature* **392:**497–501
Murthy, V.N. 1999. *Curr. Op. Neurobiol.* **9:**314–320
Nakata, T., Hirokawa, N. 1992. *J. Neurosci.* **12:**2186–2197
Naraghi, M., Neher, E. 1997. *J. Neurosci.* **17:**6961–6973
Neher, E., Zucker, R. 1993. *Neuron* **10:**21–30
Neher, E. 1998. *Neuron* **20:**389–399
Neves, G., Lagnado, L. 1999. *J. Physiol.* **515:**181–202
Ölveczky, B.P., Periasamy, N., Verkman, A.S. 1997. *Biophys. J.* **73:**2836–2847
Oheim, M., et al. 1998. *Eur. Biophys. J.* **27:**83–98
Oheim, M., et al. 1999*a. Eur. Biophys. J.* **28:**91–101
Oheim, M., et al. 1999*b. Phil. Trans. R. Soc. Lond. B* **354:**307–318
Oheim, M., Stühmer, W. 2000. *Eur. Biophys. J.* **29:**67–89
Oheim, M., 2000. Lasers in Medical Science (*in press*)
Parsons, T.D., et al. 1995. Cold Spring Harbor Symposia on Quantitative Biology **60:**389–396
Parsons, T.D. 1996. *Neuron* **15:**1085–1096
Parsons, T.D., et al. 1999. *J. Neurophysiol.* **81:**2696–2700
Pawley, J.B. 1995. Handbook of Biological Confocal Microscopy. Plenum, New York, London
Penner, R., Neher, E., Dreyer, F. 1986. *Nature* **324:**76–78
Pieribone, V.A., et al. 1995. *Nature* **375:**493–497
Plattner, H., Artalejo, A.R., Neher, E. 1997. *J. Cell Biol.* **139:**1709–1717
Pollok, B.A., Heim, R. 1999. *Trends Cell Biol.* **9:**57–
Rahamimoff, R., Fernandez, J.M. 1997. *Neuron* **18:**17–27
Reist, N.E., et al. 1998. *J. Neurosci.* **18:**7662–7673
Robinson, I.M., et al. 1995. *Proc. Natl. Acad. Sci. USA* **92:**2474–2478
Robinson, I.M., et al. 1996. *Cell Calcium* **20:**181–201
Rosenmund, C., Stevens, C.F. 1996. *Neuron* **16:**1197–1207
Rothman, J.E. 1996. *Protein Sci.* **5:**185–194
Rothman, J.E., Söllner, T.H. 1997. *Science* **276:**1212–1213
Rüden, L.v., Neher, E. 1993. *Science* **262:**1061–1065
Ryan, T.A., Ziv, N.E., Smith, S.J. 1996. *Neuron* **17:**125–134
Ryan, T.A., Reuter, H., Smith, S.J. 1997. *Nature* **388:**478–482
Sabatini, B.L., Regehr, W.G. 1998. *Biophys. J.* **74:**1549–1563
Saxton, M.J. 1993. *Biophys. J.* **64:**1766–1780
Scheller, R.H. 1995. *Neuron* **14:**893
Schneggenburger, R., Meyer, A.C., Neher, E. 1999. *Neuron* **23:**399–409
Schroeder, T.J., et al. 1994. *J. Biol. Chem.* **269:**17215–17220
Smith, C. 1999. *Neuroscience* **19:**589–598
Smith, C., Betz, W.J. 1996. *Nature* **380:**531–534
Smith, C., et al. 1998. *Neuron* **20:**1243–1253
Steyer, J.A., Horstmann, H., Almers, W. 1997. *Nature* **388:**474–478
Steyer, J.A., Almers, W. 1999. *Biophys. J.* **76:**2262–2271
Südhof, T.C. 1995. *Nature* **375:**645–653
Südhof, T.C., Rizo, J. 1996. *Neuron* **17:**379–388
Sund, S.E., Swanson, J.A., Axelrod, D. 1999. *Biophys. J.* **77:**2266–2283
Sutton, R.B., et al. 1998. *Nature* **395:**347–352
Tahara, M., et al. 1998. *J. Biol. Chem.* **273:**3367
Tan, Y.P., et al. 1999. *J. Neurosci. Methods* **92:**123–135
Thomas, P., et al. 1993. *Neuron* **11:**93–104
Thomas-Reetz, A., DeCamilli, P. 1994. *FASEB J.* **8:**209–216
Trifaró, J.-M., Vitale, M.L. 1993. *TINS* **16:**466–472
Tucker, T., Tettiplace, R. 1995. *Neuron* **15:**1323–1335
Vitale, M.L., et al. 1991. *J. Cell Biol.* **113:**1057–1067
Vitale, M.L., Seward, E.P., Trifaró, J.-M. 1995. *Neuron* **14:**353–363
Wacker, I., et al. 1997. *J. Cell Sci.* **110:**1453–1463
Weber, T., et al. 1998. *Cell* **92:**759–772
Weis, S., Schneggenburger, R., Neher, E. 1999. *Biophys. J.* **77:**2418–2429
Weiss, S. 1999. *Science* **283:**1676–1683
Xu, T., et al. 1998. *Nature Neurosci.* **1:**192–200
Zenisek, D.P., Steyer, J.A., Almers, W. 1999. *Soc. Neurosci. Abst.* **25:**1251
Zenisk, D.P., Steyer, J.A., Almers, W. 2000. *Nature* **406:**849–854
Zimmerberg, J., et al. 1999. *J. Physiol.* **520:**15–21
Zucker, R.S. 1996. *Neuron* **17:**1049–1055
Zucker, R.S. 1999. *Curr. Opin. Neurobiol.* **9:**305–313