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Exocytosis: A Molecular and Physiological Perspective

Review

Robert S. Zucker
Division of Neurobiology
University of California
Berkeley, California 94720–3200

From 1952 to about 1985, exocytosis in neurons was studied mainly at neuromuscular junctions and selected giant synapses by electrophysiology and electron microscopy. Early advances were followed by a period of relative stagnation as these techniques appeared to reach their full potential. Then, in the last few years, everything changed. New methods for measuring and controlling presynaptic calcium concentration ($[Ca^{2+}]_i$) using fluorescent dyes and photosensitive Ca^{2+}-binding compounds emerged, as did techniques for simulating the diffusion of Ca^{2+} in the presynaptic neuron. New methods of monitoring secretion were developed, including measurement of presynaptic membrane area by means of membrane capacitance, and of uptake and release by means of fluorescent dyes trapped in endocytosed vesicles. Modern methods of membrane biology and genetic engineering were applied to proteins thought to be involved in neurosecretion. Meanwhile the patch clamp permitted electrophysiological analysis of previously inaccessible tissues. As a result, exocytosis emerged from a backwater in relative obscurity to become a new frontier in biological research. This review summarizes some of the recent advances and indicates current directions of research in this active field.

Ca^{2+} and Secretion: Cooperativity, Domains, and Shells

At fast synapses, exocytosis requires high $[Ca^{2+}]_i$. Photolysis of the light-sensitive caged Ca^{2+} compound DM-nitrophen has been used to elevate $[Ca^{2+}]_i$ rapidly while monitoring secretion postsynaptically or by electronically measuring changes in presynaptic capacitance under whole-cell voltage clamp. In goldfish retinal bipolar neurons, Heidelberger et al. found that $[Ca^{2+}]_i$ had to rise to over 50 μM before the rates of secretion matched those achieved by depolarization (Heidelberger et al., 1994). At the crayfish neuromuscular junction, a transient rise in $[Ca^{2+}]_i$ to about 75 μM triggered neurosecretion with magnitude and kinetics similar to the phasic release accompanying an action potential (Landò and Zucker, 1994). These experiments demonstrated directly a sharply nonlinear dependence of secretory rate on the level of $[Ca^{2+}]_i$, and suggest that at least four Ca^{2+} ions must bind with positive cooperativity and affinities from 10–150 μM to a target complex to trigger rapid secretion. This is a significant elaboration of the original proposal of Ca^{2+} cooperativity by Dodge and Rahamimoff (1967), based on the nonlinear dependence of transmitter release on extracellular $[Ca^{2+}]$. However, other schemes are also possible. For example, Vogel et al. (1996) propose that secretion is accelerated by the Ca^{2+}-dependent activation of vesicular fusion complexes, where each vesicle contains a rather large but random number (about ten on average) of such complexes. This hypothesis can account quantitatively for the highly nonlinear Ca^{2+}-dependence of rates and extents of secretion of cortical granules in sea urchin eggs, where live exocytosis of single granules can be visualized in isolated membrane patches from these eggs.

At synapses between nerve cells, high concentrations of $[Ca^{2+}]_i$ occur only during action potentials and are localized at active zones where vesicles are docked in the neighborhood of clusters of Ca^{2+} channels. Evidence for the existence of such "Ca^{2+} microdomains" has come from three types of experiments: 1) Simulations of Ca^{2+} diffusion from Ca^{2+} channel clusters in the presence of a slowly diffusible buffer predict a complex pattern of peaks and troughs of $[Ca^{2+}]_i$ transients in active zones during action potentials (Figure 1). In these simulations, $[Ca^{2+}]_i$ reaches about 150 μM in the spaces between rows of Ca^{2+} channels where vesicles are docked. These predictions have been confirmed in actual experiments on frog saccular hair cells by measuring the activity of Ca^{2+}-activated potassium channels that are co-localized with presynaptic Ca^{2+} channels (Roberts, 1994). 2) Local Ca^{2+} domains also have been observed in turtle cochlear hair cells by confocal imaging of fast-binding low affinity fluorescent Ca^{2+} buffers (Tucker and Fettiplace, 1995) and at squid giant synapses by imaging phosphorescence from low affinity photoproteins (Llinás et al., 1995). 3) Secretion is often triggered by Ca^{2+} entering through mixtures of N- and P/Q-type high-voltage-activated Ca^{2+} channels, assessed by actions of specific channel blockers on transmission (Wheeler et al., 1994; Mintz et al., 1995). The nonlinear dependence of spike-evoked secretion on the number of channels opened indicates that secretion is triggered by Ca^{2+} microdomains arising from multiple clustered Ca^{2+} channels.

These results from neurons contrast with those obtained from endocrine cells. In endocrine cells, secretion can be measured on a fast time scale by either microamperometric detection of biogenic amines with carbon fiber electrodes or by changes in membrane capacitance. In these cells, a higher Ca^{2+}-sensitivity than in neurons (Heinemann et al., 1994), a lower maximal rate of secretion during depolarizations (Chow et al., 1994), and a persistent secretion, which outlasts Ca^{2+} influx (Chow et al., 1996), have been observed. All of these properties indicate a loose coupling of Ca^{2+} channels to the secretory machinery of endocrine cells, with exocytosis being triggered by a submembrane shell with a concentration of $[Ca^{2+}]_i$ of only about 5 μM.

Short-Term Synaptic Enhancement: Facilitation, Augmentation, and PTP

Following one or more presynaptic action potentials, transmitter release per spike is often increased by a number of processes differing in their characteristic durations—facilitation lasting about 1 s, augmentation lasting about 30 s, and potentiation or posttetanic potentiation (PTP) lasting several minutes. It has long been

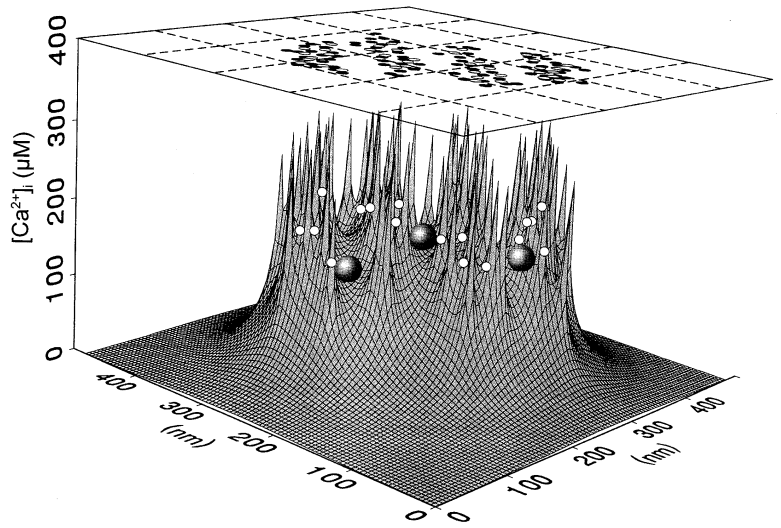

Figure 1. Simulation of a $[Ca^{2+}]_i$ Microdomain

The calculated spatial distribution of time-averaged submembrane $[Ca^{2+}]_i$ is shown for a depolarized active zone in a frog saccular hair cell. The locations of Ca^{2+} channels (solid circles) and Ca^{2+}-dependent K^+ channels (open circles) are shown on a projection above the $[Ca^{2+}]_i$ profile. The predicted average level of $[Ca^{2+}]_i$ was confirmed by measurement of Ca^{2+}-dependent K^+ current. Spheres show the locations of docked synaptic vesicles in the troughs of $[Ca^{2+}]_i$ between rows of Ca^{2+} channels. Adapted from Roberts, 1994.

thought that these effects of repeated presynaptic action potentials were the result of presynaptic accumulation of residual $[Ca^{2+}]_i$ during conditioning stimulation (Katz and Miledi, 1968; Magleby and Zengel, 1982). Recent evidence confirms this hypothesis: there is a close correlation between the decay of residual free $[Ca^{2+}]_i$ and of each process (augmentation and PTP in Aplysia central synapses and crayfish NMJ: Kretz et al., 1982; Connor et al., 1986; Delaney et al., 1989; Delaney and Tank, 1994; and facilitation in cerebellar synapses: Atluri and Regehr, 1996). Furthermore, residual $[Ca^{2+}]_i$ can be eliminated rapidly by photolysis of diazo-2, which releases a BAPTA-like Ca^{2+} chelator. At the crayfish neuromuscular junction, this maneuver reduces all three forms of synaptic enhancement (Kamiya and Zucker, 1994). It was originally thought that residual Ca^{2+} acted at the site triggering exocytosis to enhance release by simply adding to the transient rise in $[Ca^{2+}]_i$ occurring during conditioned action potentials (Miledi and Thies, 1971). But the many-fold increase in transmission often observed during facilitation, augmentation, and PTP cannot arise from such a mechanism (Yamada and Zucker, 1992) because residual $[Ca^{2+}]_i$ is usually below 1 μM while the transient rise in $[Ca^{2+}]_i$ required to trigger exocytosis during action potentials reaches 100 μM or more in active zones. This indicates that short-term synaptic plasticity is due to Ca^{2+} acting on targets distinct from the molecular Ca^{2+}-receptor triggering phasic secretion. Unlike the low affinity target(s) involved in triggering fast exocytosis, the large efficacy of low $[Ca^{2+}]_i$ levels observed during the decay of facilitation, augmentation, and PTP suggests that there must be one or more targets with high Ca^{2+} affinity.

Short-term enhancement of phasic release underlying PSPs is accompanied by a concurrent increase in spontaneous release rate or frequency of miniature postsynaptic potentials (mPSPs), often called asynchronous release. This may reflect Ca^{2+} action at one of the sites involved in short-term synaptic plasticity. Indeed, a high affinity site relating $[Ca^{2+}]_i$ to mPSP frequency is indicated when $[Ca^{2+}]_i$ is elevated to about 1 μM by Ca^{2+}-transporting ionophores (Delaney and Tank, 1994; R. Ravin et al., unpublished data) or by steady weak photolysis of DM-nitrophen (Kamiya and Zucker, 1994). Substitution of Sr^{2+} for Ca^{2+} depresses phasic release while increasing asynchronous release and facilitation (Goda and Stevens, 1994), suggesting that facilitation and asynchronous release reflect a different target of Ca^{2+} action from phasic exocytosis. This Ca^{2+} target, like that involved in phasic secretion, seems to require cooperative action of Ca^{2+} for its activation. It is interesting that at goldfish bipolar synapses, micromolar levels of $[Ca^{2+}]_i$ fail to trigger any detectable secretion, as monitored by presynaptic capacitance change (Heidelberger et al., 1994). Either these tonically transmitting synapses lack the sorts of short-term synaptic plasticities described at phasic synapses, or perhaps a low rate of secretion is obscured by simultaneous endocytosis (see Vesicle Pools below).

Flash photolysis of diazo-2 rapidly reduces residual $[Ca^{2+}]_i$ and in so doing eliminates most facilitation equally rapidly. Therefore, facilitation must be due to Ca^{2+} binding and activating its target with rapid kinetics. When residual Ca^{2+} is chelated with EGTA-AM in cerebellar synapses (Atluri and Regehr, 1996), the decay time constant of facilitation is shortened from 200 ms to 40 ms. In these synapses, 40 ms may be the decay rate of the facilitation process itself when it is activated only by the brief local rise in $[Ca^{2+}]_i$ during an action potential, while the duration of facilitation under normal circumstances is determined by the time it takes to remove residual $[Ca^{2+}]_i$ from these nerve terminals. At the crayfish neuromuscular junction, diazo-2 photolysis also eliminates augmentation and PTP, some of it rapidly but most with slow kinetics (time constant about 350 ms). The similar kinetics and sensitivities of augmentation and PTP to residual $[Ca^{2+}]_i$ (about ten-fold enhancement per μM residual $[Ca^{2+}]_i$; Delaney and Tank, 1994), and the fact that facilitation and augmentation/PTP interact multiplicatively (Magleby and Zengel, 1982) suggest that augmentation/PTP are due, at least in part, to Ca^{2+} acting at a site with higher affinity and slower kinetics than the site triggering facilitation. The prolonged persistence of PTP after long tetani is partly

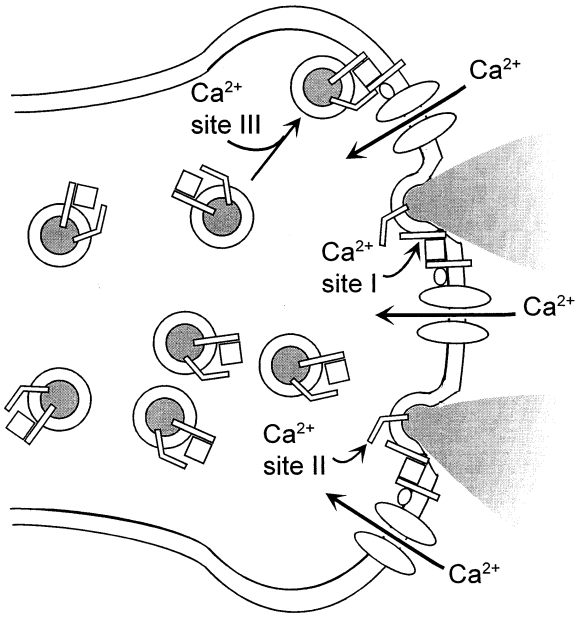

Figure 2. Possible Sites of Ca^{2+} Action in Exocytosis and Short-Term Synaptic Plasticity

Undocked, docked, and exocytosing synaptic vesicles are shown in a synaptic terminal, along with open Ca^{2+} channels admitting Ca^{2+} into the terminal. Exocytosis is triggered by Ca^{2+} binding at site I, perhaps to synaptotagmin I or affecting the interaction between Ca^{2+} channels and syntaxin or SNAP-25. Facilitation is activated by Ca^{2+} ions at site II, which may be another vesicular protein such as synaptotagmin III or VII. Augmentation/PTP is caused by Ca^{2+} acting at site III, which may be a vesicular or cytoplasmic protein involved in docking vesicles at active zones.

due to presynaptic Na^+ accumulation and consequent retardation of Ca^{2+} removal by Na^+/Ca^{2+} exchange (Mulkey and Zucker, 1992), and partly to posttetanic leakage of Ca^{2+} from mitochondria that are loaded only during long tetani (Y.-G. Tang and R.S. Zucker, in preparation).

Where Are These Ca^{2+} Targets Located That Are Involved in Exocytosis, Facilitation, and Augmentation/PTP?

The shortness of the synaptic delay, 0.2 ms from the opening of Ca^{2+} channels to the beginning of transmitter release (Llinás et al., 1981), requires that exocytosis be triggered by Ca^{2+} acting in the immediate neighborhood (within tens of nanometers) of Ca^{2+} channel clusters. This locates the exocytotic targets of Ca^{2+} to membrane proteins near Ca^{2+} channels or to proteins in nearby docked vesicles (see Molecular Components below). Since facilitation is maximal shortly after an action potential, Ca^{2+} must be acting reasonably near Ca^{2+} channels, perhaps on a different protein in the docking/fusion complex, a protein involved in attaching vesicles to the plasma membrane. The slow kinetics of augmentation/PTP allow for the involvement of second messengers, and even for such processes as the movement (or mobilization, as it is called) of predocked vesicles to docking sites at the plasma membrane (Figure 2).

What are the functions of short-term synaptic enhancement in the neural processing of information? Synaptic transmission which requires facilitation, augmentation, or PTP to reach postsynaptic threshold for generating action potentials, produces an effective filter that responds only to sustained high frequencies; this property can be used to extract signals from noise. Two more concrete applications of synaptic plasticity have recently come to light. In the first, the polysynaptic component of the Aplysia siphon withdrawal reflex habituates to repeated tactile stimulation, and this behavior is due to PTP in an inhibitory interneuronal feedback circuit, which progressively reduces the gain of excitatory interneuronal input to the motor neurons (Fischer and Carew, 1993). In the second, computer simulations of the activity within hypothetical neuronal circuits show that inclusion of facilitation in selected synapses permits the circuit to transform different temporal patterns of afferent activity into distinct spatial patterns of neuronal activation (Buonomano and Merzenich, 1995). Recently, experimental evidence has been obtained for just such transformations in the CA3 region of the hippocampus (D. V. Buonomano, P. W. Hickmott, and M. M. Merzenich, in preparation).

Is Fusion Always Complete or Can It Be Transient?

Neurosecretion might involve complete fusion of vesicles with the plasma membrane and subsequent endocytosis, or a transient "kiss-and-run" fusion could allow vesicle contents to escape through a briefly opened fusion pore (Figure 3). Early ultrastructural and tracer uptake studies provide support for both modes of operation under different stimulus conditions. More recent evidence for complete fusion comes from staining and subsequent destaining of internalized membranes in nerve terminals with FM1–43 during electrical stimulation. The finding that staurosporine can abolish FM1–43 destaining and slow down staining with little effect on transmitter release (Henkel and Betz, 1995) suggests that secretion at frog motor nerve terminals can switch between complete and transient fusion modes.

Analysis of rise times of mPSPs at the frog neuromuscular junction indicates that acetylcholine escapes through a fusion pore similar to that measured electrically during fusion of mast cell granules (van der Kloot, 1995), rather than by instantaneous complete fusion of vesicles with the surface membrane. A lack of effect of monovalent and divalent cations on rise time of mPSPs indicates that, unlike the release of serotonin from mast cells, acetylcholine may not be released from an intravesicular ion exchange matrix. Finally, amperometric measurements of release of serotonin from cultured leech neurons allowed measurement of the amount and kinetics of quantal release (Bruns and Jahn, 1995). Small responses presumably arising from fusion of small vesicles were due to release of about 5,000 molecules within 100 μs, consistent with a rapid opening of a preassembled fusion pore. Large responses presumably arising from fusion of large dense core vesicles were due to release of about 80,000 molecules through a pore that

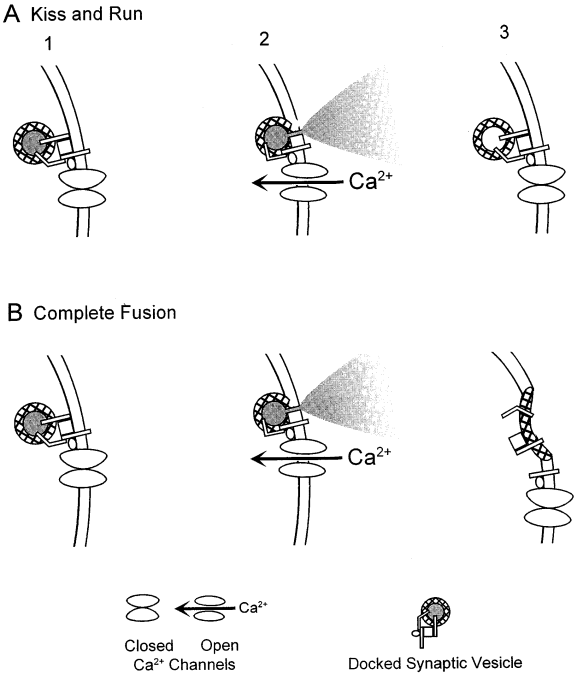

Figure 3. Two Modes of Exocytosis

In "kiss-and-run," transmitter is released through a transient opening of a fusion pore from the vesicle through the plasma membrane. In "complete fusion," the vesicle membrane fuses with and becomes embedded in the plasma membrane following release of transmitter.

opened initially like that of small vesicles but then progressively dilated. It is not yet clear whether in most circumstances this pore subsequently closes or dilates fully to produce complete fusion.

Vesicle Pools

Much attention has been focused on pools of transmitter that are to varying degrees available or competent for release. Classically, this question has been approached by attributing different kinetic components of depression of repeated PSPs to depletion of transmitter stores successively more distant from release sites. Recently, release has been activated by continuous depolarization, hypertonic stimulation, or steps of $[Ca^{2+}]_i$ imposed by photolysis of caged Ca^{2+} compounds, while secretion has been monitored by mPSP frequency, presynaptic capacitance increase, or FM1–43 destaining. Often an early phase of rapid secretion occurs, followed by one or more slower phases, and in "fire and reload" models of secretion, these are interpreted as reflecting release of fully docked and primed vesicles followed by (a reloading and) release of vesicles progressively less competent for fusion.

For example, chick retinal amacrine cells subjected to depolarizing pulses release 0 or 1 quantum, equal in size to a spontaneous mPSP, from each of 10 release sites at a rate of 150/s/site (with a decay constant of depleting this store, $\tau = 7$ ms). Subsequent release occurs at 22/s/site from a much larger store (Borges et al., 1995). Rat pituitary synaptosomes initially release 40 vesicles to a depolarization ($\tau = 14$ ms), and then release another 190 vesicles more slowly ($\tau = 430$ ms, Hsu and Jackson, 1996). Goldfish retinal bipolar cells contain 55 ribbon synapses, to each of which are attached 100 vesicles, 20 of them docked at the membrane (von Gersdorff et al., 1996). The 1100 docked vesicles (20×55) can be released rapidly ($\tau = 0.5$ ms) by depolarization, and this pool is refilled after depolarization with a 5 s time constant (Mennerick and Matthews, 1996, [this issue of *Neuron*]). The remaining 5000 ribbon vesicles are released more slowly ($\tau = 300$ ms) during prolonged depolarization, perhaps reflecting the rate at which these can replace the docked vesicles. Since this is faster than the 5 s recovery rate for the fast release of docked vesicles at rest, and since presynaptic perfusion of EGTA blocks the slow component during persistent depolarization, Ca^{2+} may mobilize vesicles to docking sites at the membrane.

In bovine chromaffin cells, Ca^{2+} also appears to mobilize vesicles (Neher and Zucker, 1993; von Rüden and Neher, 1993) from a reserve pool of about 1000 ($\tau = 500$ ms) to a fully releasable pool of 100–200 (emptiable with $\tau = 2$ ms, Heinemann et al., 1994). In chromaffin cells and melanotrophs, the immediately releasable and fully primed pool corresponds to about 10–20% of morphologically docked vesicles (Parsons et al., 1995). In contrast to these examples, frog saccular hair cells appear capable of sustained high levels of quantal release—500/s/active zone for up to 2 s without apparent depletion—despite the fact that in active zones (defined as having a dense body) only about 30 of 80 vesicles are docked at the membrane (Parsons et al., 1994).

These studies raise again the important, but still unresolved question of whether mobilization of vesicles from reserve pools to docking sites, or the priming of docked vesicles, correspond to particular phases of short-term synaptic enhancement such as facilitation or augmentation/PTP, as proposed in Figure 2.

In most of these studies, secretion was monitored as an increase in membrane capacitance, and a rise in capacitance to a new steady level was taken as indicating secretion occurring during the rise. A final flat trace is assumed to represent cessation of exocytosis, but it could equally well represent the delayed onset of a balanced rate of endocytosis. Simultaneous use of capacitance and either amperometry or FM1–43 staining (Chow et al., 1994, 1996; Smith and Betz, 1996) allow distinction between these alternatives. At salamander rod photoreceptor synapses, a sudden elevation of $[Ca^{2+}]_i$ by photolysis of caged Ca^{2+} compounds led to a step rise in capacitance, while sudden reduction in $[Ca^{2+}]_i$ by photolysis of the caged Ca^{2+}-absorbing chelator diazo-2 led to a step drop, showing that the resting steady capacitance represents continuous balanced rates of exo- and endocytosis (Rieke and Schwartz, 1996).

In cultured rat hippocampal neurons, single boutons containing one active zone can be activated by focal perfusion of hyperosmotic solutions or solutions containing high-K^+/high-Ca^{2+}. Single 5 s stimuli (Stevens and Tsujimoto, 1995), as well as trains of action potentials (Rosenmund and Stevens, 1996), evoke release of a readily releasable pool of 15 quanta at 20/s; this pool can be replenished with a 10 s time constant. One second hyperkalemic stimuli repeated at 0.2–0.5 Hz, which

permit replenishment of this pool, release 90 quanta, corresponding to the total number of vesicles in a bouton; refilling this pool takes about 40 s (Liu and Tsien, 1995). This corresponds to the time required for endocytosis of released vesicles and their reentry into a releasable pool, as measured by FM1–43 destaining (Ryan and Smith, 1995). In this preparation, action potentials only release one quantum per release site, release occurs only about 25% of the time, and release sites remain refractory for 5–20 ms afterwards (Allen and Stevens, 1994; Stevens and Wang, 1995). If a quantal PSP is due to release of 1 vesicle, this would correspond to a vesicle depletion rate of 1 per 4 spikes or 0.25% of the total vesicle pool. This is close to estimates of the rate of vesicle depletion per action potential from FM1–43 destaining experiments (Ryan and Smith, 1995), and this correspondence is one of the strongest arguments for the common assumption that single vesicles are responsible for the quantal components of transmitter release.

At the squid giant synapse, photolysis of caged Ca^{2+} generates a step rise in $[Ca^{2+}]_i$ but only a transient postsynaptic depolarization reflecting secretion decaying with a time constant of 30 ms (Hsu et al., 1996). A series of step rises in $[Ca^{2+}]_i$ led to a series of transient releases of transmitter. Many of the results can be quantitatively explained by depletion of an immediately releasable transmitter pool, with a compensatory Ca^{2+}-dependent mobilization into that pool (R. Zucker, unpublished data). Hsu et al. prefer an alternative interpretation, that the secretory process displays a sort of adaptation in response to step rises in $[Ca^{2+}]_i$. If the latter interpretation is correct, the Ca^{2+}-dependence of secretion is likely to prove even more complex than is now believed.

Molecular Components

Molecular biology has allowed the identification and cloning of a number of proteins involved in vesicle docking and fusion. Some synaptic vesicle proteins (synaptotagmin and synaptobrevin) form tight associations with plasma membrane proteins (syntaxin, SNAP-25, and N-type Ca^{2+} channels) to form a protein complex called the fusion complex. Other vesicle or soluble proteins (NSF, α-SNAP, and munc18 or n-sec1) appear to be involved in assembling the complex. Several of these proteins are homologous to yeast proteins involved in membrane trafficking and fusion. Important evidence for their involvement in neurosecretion came from the inhibition of secretion by proteolytic fractions of botulinum and tetanus neurotoxins that specifically cleave synaptobrevin, syntaxin, and SNAP-25, and from the discovery that transmission is inhibited by presynaptic injection of peptides that compete with the action of synaptotagmin, synaptobrevin, and α-SNAP (Calakos and Scheller, 1996).

Of all these proteins, synaptotagmin has received the most attention recently. Containing two C2 Ca^{2+}-binding domains and being part of the fusion complex, it is an obvious candidate for transducing an elevation in $[Ca^{2+}]_i$ into a secretory trigger. The C2A domain of synaptotagmin I shows Ca^{2+}-dependent binding to syntaxin and membrane phospholipids (apparent K_Ds of about 300 and 5 μM, respectively) that may be a first step in triggering membrane fusion (Li et al., 1995). Presynaptic injection of fragments of the C2 domains (which might act as competitive blockers of the action of synaptotagmin) blocks transmitter release without altering Ca^{2+} influx or vesicle docking (Bommert et al., 1993). Release is also blocked by polyclonal antibodies made to a fragment that includes the C2A domain, which shows Ca^{2+}-dependent binding, but not antibodies to the C2B domain, which does not bind Ca^{2+} (Mikoshiba et al., 1995). Cultured hippocampal neurons from mice with synaptotagmin I deletion mutants show a specific defect in phasic transmitter release (Geppert et al., 1994). The synchronized release of quanta immediately following an action potential that constitutes the fast PSP was absent, but asynchronous release, the delayed increase in mPSP frequency following action potentials, persisted. This is consistent with synaptotagmin acting either as a Ca^{2+}-sensitive activator of release or as a Ca^{2+}-inhibited brake on spontaneous or constitutive release. It will be interesting to see if these defects can be rescued by replacement of synaptotagmin or by use of conditional knockouts.

Synaptotagmin is not, however, the only molecule associated with vesicle fusion showing a Ca^{2+}-sensitivity. N-type Ca^{2+} channels bind separately to syntaxin and to SNAP-25 independently of synaptotagmin; this binding is strongly activated by increasing $[Ca^{2+}]$ to 20 μM, and inhibited at higher $[Ca^{2+}]$ (Sheng et al., 1996). This biphasic Ca^{2+}-dependence could underlie a dual-phase "cock-and-fire" triggering of secretion by Ca^{2+}. Interactions in the reverse direction also exist (Bezprozvanny et al., 1995): syntaxin coexpressed in oocytes with N- and Q-type Ca^{2+} channels stabilizes channel inactivation. A fragment of the protein binding region of L-type Ca^{2+} channels dissociates the channels from syntaxin and inhibits phasic transmitter release following action potentials without altering Ca^{2+} influx when injected into cultured sympathetic neurons (Mochida et al., 1996); asynchronous release and synaptic facilitation, however, were enhanced. These effects, similar to those seen in synaptotagmin deletion mice, could reflect the disruption of the tight association of Ca^{2+} channels with their molecular targets, removing the targets from the shoulders of Ca^{2+} microdomains and leaving them in the valleys where release is triggered only weakly and asynchronously by Ca^{2+} influx (Figure 1). In this view, synaptotagmin might be the target of these Ca^{2+} microdomains, but so might other Ca^{2+}-binding proteins such as rabphillin, doc2, or scinderin (Calakos and Scheller, 1996; Zhang et al., 1996). Alternatively, the Ca^{2+} sensor in secretion could be the channel-syntaxin or channel-SNAP-25 interaction, and synaptotagmin mutants or inhibitors might disrupt this interaction. Thus, the evidence to date does not point uniquely to a single candidate as the Ca^{2+} target in neurosecretion (site I in Figure 2).

It is possible that the increased asynchronous release and synaptic facilitation observed on dissociation of Ca^{2+} channels from syntaxin and in synaptotagmin mutants reflect a separate target of Ca^{2+} action in these processes, as proposed above (site II in Figure 2). Candidates for this action include the neural synaptotagmins III and VII, since they show a much higher (micromolar) Ca^{2+}-sensitivity in syntaxin binding than does synaptotagmin I (Li et al., 1995).

It has been suggested that kinetically slow augmentation and PTP are due to Ca^{2+} acting via Ca^{2+}/calmodulin-dependent kinase II (CaMKII) to phosphorylate synapsin I and "mobilize" vesicles tethered to actin microfilaments at release sites (site III in Figure 2; Greengard et al., 1993). Presynaptic injection of antibodies to synapsin I leads to a depression of high-frequency transmission and a depletion of vesicles at release sites (Pieribone et al., 1995). But mice with synapsin I and CaMKII gene knockouts show normal or even enhanced PTP (Rosahl et al., 1995; Chapman et al., 1995), and calmodulin and CaMKII inhibitors fail to block facilitation, augmentation, or PTP (Kamiya and Zucker, 1994). Mice without synapsin II show reduced PTP, but synapsin II is not phosphorylated by CaMKII. Interestingly, facilitation is increased in synapsin I and reduced in CaMKII knockouts. The results indicate that facilitation is regulatable independently from PTP and secretion and suggest that synapsins and CaMKII can influence the rate of supply of primed vesicles available for release during repetitive stimulation but do not mediate generation of synaptic enhancement.

What's Next?

The immediate future seems to lie with the imaginative combination of techniques. Combinations with complementary advantages and compensatory limitations promise new insights into questions of how Ca^{2+} triggers secretion and synaptic plasticity, functions of components of the molecular machinery, and the detailed behavior of vesicles during secretion.

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