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Review

Multiple Roles of Calcium Ions in the Regulation of Neurotransmitter Release

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The intracellular calcium concentration ([Ca^{2+}]) has important roles in the triggering of neurotransmitter release and the regulation of short-term plasticity (STP). Transmitter release is initiated by quite high concentrations within microdomains, while short-term facilitation is strongly influenced by the global buildup of "residual calcium." A global rise in [Ca^{2+}] also accelerates the recruitment of release-ready vesicles, thereby controlling the degree of short-term depression (STD) during sustained activity, as well as the recovery of the vesicle pool in periods of rest. We survey data that lead us to propose two distinct roles of [Ca^{2+}] in vesicle recruitment: one accelerating "molecular priming" (vesicle docking and the buildup of a release machinery), the other promoting the tight coupling between releasable vesicles and Ca^{2+} channels. Such coupling is essential for rendering vesicles sensitive to short [Ca^{2+}] transients, generated during action potentials.

The intracellular [Ca^{2+}] signal has a bewildering multitude of regulatory functions, and one may ask how a single species of second messengers can be involved in so many different roles. In the nerve terminal, it is well established that the most prominent one—Ca^{2+}-triggering of neurotransmitter release—achieves its unique properties by activating a relatively low-affinity Ca^{2+} sensor. Therefore, high Ca^{2+} concentrations are required, which persist for only very short episodes (during action potentials) and are spatially constrained to the vicinity of open Ca^{2+} channels. In contrast, other functions are mediated by slow variations of [Ca^{2+}] of much smaller amplitude, which build up throughout the nerve terminal during episodes of high synaptic activity and decay during periods of rest. Such slow increases in [Ca^{2+}] accelerate the recruitment of vesicles (Dittman and Regehr, 1998; Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998), influence the release probability during action potentials, and may trigger asynchronous release. Detailed kinetic characterization of these processes is required to understand the interactions between them. We will review the current status of such studies, with the calyx of Held synapse taking a central role. This preparation provides quantitative dose-response curves for the action of calcium, as well as accurate kinetic information, since it allows for simultaneous pre- and postsynaptic voltage clamp, combined with fluorimetric Ca^{2+} measurement and caged-Ca^{2+} stimulation. We will argue that changes in synaptic properties, which go along with molecular perturbations, rarely can be interpreted as a one-to-one relationship between the given molecule and an observed functional change. In this context, we will consider the debate about multiple Ca^{2+} sensors (e.g., for synchronous and asynchronous release or else for facilitation). We will also focus on the question of what are the rate-limiting steps for sustained release and what molecules control these. We will compile evidence that the enhancement of vesicle priming by elevated [Ca^{2+}] may, indeed, reflect two mechanistically distinct steps.

Action-Potential-Induced Neurotransmitter Release Is Triggered by Microdomain [Ca^{2+}] and Is Relatively Robust

An action potential triggers a short burst of neurotransmitter release—the so-called synchronous release—followed by a decaying tail of "asynchronous release," which is about two orders of magnitude smaller in terms of vesicle release rates. Asynchronous release can build up during trains of action potentials, particularly so in some types of inhibitory neurons (Lu and Trussell, 2000; Hefft and Jonas, 2006). During prolonged pauses of activity, release rates decay back to the level of so-called spontaneous release, which gives rise to miniature postsynaptic currents.

Synchronous release can be readily explained by short-lived, so-called micro- or nanodomains of elevated [Ca^{2+}], which build up and decay rapidly around voltage-dependent Ca^{2+} channels, while these open and close during action potentials (Adler et al., 1991; Llinas et al., 1992; Stanley, 1993; Neher, 1998; Bucurenciu et al., 2008). Detailed biophysical modeling studies have explored the properties of such nanodomains in terms of a reaction-diffusion mechanism by which Ca^{2+} ions move away from Ca^{2+} channel mouths and interact with both mobile and fixed Ca^{2+} buffers (Zucker, 1996; Neher, 1998). We will use the term nanodomain for the immediate vicinity of a few Ca^{2+} channels, where Ca^{2+} is not in equilibrium with fast buffers. This is typically in the range 10–100 nm (Naraghi and Neher, 1997). Microdomains, in our terminology, include nanodomains as well as subcellular regions of elevated [Ca^{2+}] (e.g., in the vicinity of active zones, typically 100 nm to 1 μm across).

The dimensions of nanodomains are well below the diffraction limit of light microscopy, such that their properties cannot be directly recorded by optical Ca^{2+}-imaging techniques. However, quantitative experimental data on amplitude and time course of nanodomain Ca^{2+} signals have been obtained by a three-step "bioassay" approach. These studies first establish an experimental "dose-response curve," which is a relationship between

Table 1. Ca^{2+} Dependence of the Rate of Exocytosis in Various Secretory Cells

Cell Types	Max. Rate	$K_{10\%}$[a]	Delay at $K_{10\%}$	Ca Cooperativity	References
Pituitary melanotrophs	25 s^{-1}	20 μM	ND	3	1
Pancreatic β cells	70 s^{-1}	1–10 μM	ND	2–5	2, 3
Chromaffin cells	1500 s^{-1}	40 μM	3 ms	3	4
Rod photoreceptors	300 s^{-1} (?)	10 μM (?)	ND	3	5
Bipolar cells	3000 s^{-1}	80 μM	1 ms	4	6
Inner hair cells	1700 s^{-1}	20 μM	1.5 ms	5	7
Calyx of Held	6000 s^{-1}	30 μM	0.3–1 ms	5	8, 9
Inhibitory basket cell (cerebellum)	5000 s^{-1}	20 μM	1 ms	5	10

References: 1, Thomas et al. (1993); 2, Barg et al. (2001); 3, Wan et al. (2004); 4, Voets (2000); 5, Thoreson et al. (2004); 6, Heidelberger et al. (1994); 7, Beutner et al. (2001); 8, Bollmann et al. (2000); 9, Schneggenburger and Neher (2000); 10, Sakaba (2008).
[a] $K_{10\%}$: Ca concentration at which the rate constant is 10% of the maximal rate.

release rate and intracellular [Ca^{2+}]. To make these measurements, flash photolysis of Ca^{2+} is used to produce spatially uniform [Ca^{2+}] elevations while measuring [Ca^{2+}] fluorimetrically and measuring release rates on the basis of the postsynaptic response. In a second step, a kinetic model of the release process is formulated, which accurately describes the caged-Ca^{2+} data within the relevant range of [Ca^{2+}]. In a third step, the time course of release during an AP is measured, and it is asked which [Ca^{2+}] waveform and magnitude have to be postulated, such that the kinetic model would reproduce the AP-induced release. Caged-Ca^{2+} studies of release kinetics have been performed on a number of preparations, ranging from neuroendocrine cells to ribbon synapses and synapses of the central nervous system (see Table 1). Usually, peak release rates following step-like increases in [Ca^{2+}] were evaluated, as well as a parameter describing the delays of responses. With few exceptions, it was found that peak vesicle release rates rise within a narrow [Ca^{2+}] range to values in the range 10^2 to 10^4 (vesicle × s)$^{-1}$, which means that the mean survival time of a release-ready vesicle (before exocytosing), if exposed to high [Ca^{2+}], is between 10 ms and a fraction of a millisecond (see Table 1). Relationships between release rates and [Ca^{2+}] were approximated by power laws with exponents between 3 and 6. In two studies at the calyx of Held, the relationship was extended to very low [Ca^{2+}] (Lou et al., 2005; Sun et al., 2007). Both studies found that for release rates lower than 1 μM the power law no longer provided an accurate description. Rather, it was found that an allosteric model can be applied, in which Ca^{2+} binding to five subunits of a release machine increased release rates in a cooperative fashion. The dynamic range of this Ca^{2+}-dependent action was about seven orders of magnitude, increasing the spontaneous release rate of 0.45 vesicles/s to maximum values around 2 × 10^6 vesicles/s (Lou et al., 2005). The other study assigned the deviations from the simple power law to the contribution of a second high-affinity Ca^{2+} sensor (Sun et al., 2007).

Amplitudes of [Ca^{2+}] microdomains and their temporal half-widths (Bollmann et al., 2000; Schneggenburger and Neher, 2000) as derived by step three of the "bioassay approach" are well compatible with the expectations of a diffusional model, if it is assumed that mean distances between vesicles and nearby channels are 30–60 nm (Meinrenken et al., 2002). The presumed short lifetime of [Ca^{2+}] microdomains was corroborated by an elegant study in which [Ca^{2+}] transients (rather than step-like [Ca^{2+}] elevations) were produced by photolysis of caged Ca^{2+} (Bollmann and Sakmann, 2005). It turned out that only [Ca^{2+}] waveforms as short as those predicted were able to reproduce typical EPSC waveforms. Any transients longer than that would result in excessively long rise times of EPSCs. The microdomain properties were also explored by confocal spot measurements. In this technique, light from a laser-illuminated source pinhole was projected through the epi-illumination port of the microscope and focused to a submicrometer "spot" on the specimen (DiGregorio et al., 1999). [Ca^{2+}] hotspots of micrometer dimension were detected, which most likely represent the convolution of even smaller microdomains with the point spread function of the microscope objective.

Given the fact that the release machinery is made up of a large number of synaptic proteins (i.e., the SNAREs, SM-proteins, complexin, synaptotagmins), many of which may be modulated by phosphorylation and other influences, one would expect that the release process itself should be subject to multiple modulation and quite sensitive to experimental perturbations. However, only a few "modulators" have been found, so far, which would clearly change the properties of the intracellular dose-response curve, such as its Ca^{2+} sensitivity, steepness, maximum rate, etc. One such modulator is phorbol ester, which activates Munc13 and PKC (Betz et al., 1998; Wierda et al., 2007). A careful study at the calyx of Held showed that phorbol esters raise the low end of the dose-response curve, together with increasing spontaneous release (Lou et al., 2005). Remarkably, in the framework of an allosteric model of release, the dose-response curve of the control case could also describe the phorbol ester data after the increase of only a single parameter, which is the spontaneous release rate in the Ca^{2+}-unbound (or relaxed) state. In endocrine secretion, phorbol esters were shown to shift the dose-response curve (Wan et al., 2004) or else to bring forward a component of release, which is ultrasensitive toward [Ca^{2+}] (Yang and Gillis, 2004).

Another group of "modulators" are the clostridial neurotoxins, which have long been known to target the core of the release machinery—the SNARE proteins (Blasi et al., 1993; Chen and Scheller, 2001; Schiavo et al., 2000). In a caged-Ca^{2+} study at

the calyx of Held (Sakaba et al., 2005), it was found that two toxins, which cleave syntaxin (BoNTxC) and synaptobrevin (TetTx), progressively reduce release while leaving the kinetics of the remaining vesicles unchanged. In contrast, a toxin that removes a small peptide from SNAP25 (BoNTxA) strongly altered the dose-response curve and the kinetics of release. These results were readily explained by the assumption that a single hit by BoNTC and TetTx renders the release machinery with its corresponding vesicle nonfunctional, while the slight modification of SNAP25 by BoNTxA has less drastic effects, altering energetics and kinetics of the response, but not eliminating it completely. Likewise, in adreneal chromaffin cells, mutations in SNARE proteins, which would interfere with the zippering of the SNARE complex (Sorensen et al., 2006) or else with the linkage between the SNARE complex and the vesicle membrane (Kesavan et al., 2007), led to drastic changes in release kinetics. Many other mutations on SNARE proteins influenced vesicle pool sizes rather than the speed and Ca^{2+} sensitivity of the triggering reaction (reviewed by Neher, 2006).

At the calyx of Held, a massive change in the dose-response curve was induced by molecular genetics (Sun et al., 2007). Here, the gene for synaptotagmin 2—the dominant isoform of this protein at this synapse—was ablated. As a consequence, it was found that the steep rise in release rate at higher [Ca^{2+}] was lacking, while the lower end of the dose-response curve was almost unchanged, except for a higher basal value. This was interpreted in terms of the lack of the Ca^{2+} sensor for fast, synchronous release (synaptotagmin 2) and the remaining release being triggered by an independent second Ca^{2+} sensor for slow release. This is, indeed, to date the most direct evidence for such a separate sensor. However, inspection of the data of Sun et al. (2007) suggests that release in the absence of synaptotagmin 2 can also be described by an allosteric model with reduced dynamic range. It might well be that synaptotagmin 2 is specialized (like synatotagmin 1) in forming release machines of high dynamic range, while other isoforms (which would compensate for synaptotagmin 2 in its absence) would elicit more spontaneous release and enhance release rates upon binding of Ca^{2+} only to a smaller extent. Additionally, it should be pointed out that the biophysical properties of the [Ca^{2+}] signal alone are complex enough to produce synchronous and asynchronous release with just a single sensor (Xu-Friedman and Regehr, 1999). Thus, one can readily assign the rapid decay of the synchronous release to the process of diffusional collapse of nanodomains after closure of ion channels, while the slow and complex decay of asynchronous release (Scheuss et al., 2007) would reflect further diffusional collapse of microdomains, Ca^{2+} binding to slow buffers (Müller et al., 2007), and Ca^{2+} reuptake and extrusion (Kim et al., 2005). These processes operate in different kinetic regimes, since the kinetics of nanodomains are dominated by the rapid binding of Ca^{2+} to fast buffers and diffusion on very small length scales (Naraghi and Neher, 1997), which is orders of magnitude faster than long-range diffusion and Ca^{2+} sequestration.

Quite a number of additional mechanisms have been postulated to influence the Ca-triggered exocytosis, such as modulation by cAMP (Trudeau et al., 1997), inositolpolyphosphates (Illies et al., 2007), and $\beta\gamma$ subunits of G proteins (Blackmer et al., 2001, 2005; Gerachshenko et al., 2005). It should be noted, though, that these studies were based on overall assays of release and not on a direct measurement of the intracellular [Ca^{2+}] dose-response curve. Such studies very often cannot distinguish between effects on Ca^{2+} triggering and those on vesicle pool sizes. Prominent effects of $\beta\gamma$ subunits of G proteins were ascribed to interference with Ca^{2+} triggering (Blackmer et al., 2001). However, the same analogs did not produce any change in the final steps of fusion in the calyx of Held (Takahashi et al., 2000; Sakaba and Neher, 2003b). Rather, they prevented the Ca^{2+} enhancement of vesicle recruitment (to be described below).

In summary, we would like to propose that the core of the release machinery should be viewed as a relatively robust system. Quantitative differences in its properties between different secretory systems may reside in the choice of isoforms of participating proteins (e.g., synaptotagmin 2 versus synaptotagmin 1) (Nagy et al., 2006; Xu et al., 2007) or else different complexins (Xue et al., 2008), SNARES (Sorensen et al., 2003; Borisovska et al., 2005), and Munc13s (Rosenmund et al., 2002). Interference with this core by clostridial toxins, mutations, or gene ablation will, of course, change its properties. However, physiologically acting modulation of the intracellular dose-response curve has so far only been well-proven for phorbol esters and signaling pathways, which act through their natural analog, diacylglycerol.

Vesicle Docking and Priming Are Enhanced by Globally Increased Intraterminal [Ca^{2+}]

In phasic synapses, such as glutamatergic synapses in the hippocampus and in the cortex, a major cause of short-term depression (STD) during sustained activity is the partial exhaustion of a pool of release-ready vesicles (Zucker and Regehr, 2002). While other factors (e.g., postsynaptic receptor desensitization) may contribute to STD (Trussell et al., 1993; Koike-Tani et al., 2008), the steady release is determined by a balance between vesicles being consumed during APs and the recruitment of new ones. It has been shown in a variety of neuronal and neuroendocrine preparations that the recruitment process is enhanced by elevated [Ca^{2+}] (Von Rüden and Neher 1993; Dittman and Regehr, 1998; Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998), and it was recognized that this enhancement is essential for maintaining adequate release during high-frequency bursts of activity (Dittman et al., 2000).

In other types of synapses, so-called tonic synapses (Atwood and Karunanithi, 2002; Millar et al., 2005), medium-frequency bursts of activity do not lead to depression, but rather to a progressive increase in synaptic strength by up to two orders of magnitude—a process sometimes called synaptic enhancement (Mossy fiber synapse; Regehr et al., 1994) or "frequency facilitation" (lobster nmj; Worden et al., 1997). It has been debated whether this increase reflects an increase in release probability or else an increase in the availability of vesicles (i.e., an increase in the size of the release-ready vesicle pool). A recent caged-Ca^{2+} study on the crayfish neuromuscular synapse, comparing "phasic" and "tonic" synapses, which coexist in this preparation, came to the conclusion that differences between the two types can be well described if one assumes that tonic synapses have a relatively empty vesicle pool at rest (Millar et al., 2005). In

this scheme, synaptic enhancement reflects an increase of the recruitment rate, outweighing the vesicle consumption and actually leading to an increase in pool size during a burst of activity. If this is the case, and if the enhanced recruitment is due to elevated [Ca^{2+}], one would expect a transient overfilling of the pool at the end of a burst of activity due to enhanced vesicle recruitment, as long as [Ca^{2+}] stays elevated. This signature of transient overfilling has actually been observed in hippocampal autapses of transgenic animals, in which the lack of Munc13 was rescued by overexpression of Munc13-2 (Rosenmund et al., 2002). Wild-type synapses of this kind usually express mainly Munc13-1 and display net depression when stimulated with a 10 Hz train. After such trains, EPSC amplitudes recover toward the resting level. In synapses rescued with Munc13-2, a pronounced rebound effect was observed, with a peak EPSC up to 3-fold over the resting EPSC shortly after the end of a stimulus train (10 Hz). Similarly, autapses from transgenic mice lacking the CAPS protein showed a very strong rebound effect of this type (Jockusch et al., 2007).

These findings are most readily explained if one assumes a dynamic equilibrium between a reserve pool of vesicles and a primed pool, in which the filling state of a certain number of priming sites is determined by the ratio of priming and "depriming" rates (see scheme I, Figure 2). In phasic synapses, the priming rate at rest would be larger than the depriming rate, such that the priming sites would mostly be occupied; while in tonic synapses, the opposite would be the case. Enhanced priming by elevated [Ca^{2+}] would lead to an increased pool and transient overfilling in tonic synapses, but not so much in phasic synapses, in which the pool is already close to its maximum at rest. This scheme has its roots in similar phenomena observed in neuroendocrine cells. The pool of release-ready granules in adrenal chromaffin cells (Voets, 2000) and pancreatic cells (Gromada et al., 1999) shows such a dynamic equilibrium. It is increased several-fold concomitantly with an increase in recruitment rate when elevating basal [Ca^{2+}] by 100–200 nM (Voets, 2000), and it shows "depriming" within seconds when [Ca^{2+}] is lowered (see Sorensen, 2004, for review). Dynamic docking and undocking of vesicles is also observed in TIRF measurements on ribbon synapses (Zenisek et al. 2000). In the calyx of Held, strong Ca^{2+}-dependent enhancement of vesicle recruitment has been found (see below). However, changes in recruitment rate were not paralleled by changes in resting pool size, which indicates that the pool is full even under relatively low recruitment rates at resting [Ca^{2+}]. Obviously, the calyx of Held is a "phasic" synapse in the sense of the above discussion (see also Neher, 2006). Hippocampal excitatory synapses of wild-type animals would also be "phasic" ones. However, the same synapses in the absence of CAPS or when rescued with Munc13-2 would be tonic, due to a very unstable primed state, as suggested by Jockusch et al. (2007).

Facilitation, Synchrony, and Spontaneous Release

So far, our discussion of the effects of global [Ca^{2+}] elevation was restricted to the [Ca^{2+}] influence on vesicle recruitment and pool size. However, higher basal [Ca^{2+}] also is expected to have an influence on evoked release, since it will lead to higher peak [Ca^{2+}] during action potentials and, consequently, to a higher probability of release (p) during an AP. This mechanism is the basis of the "residual-calcium" hypothesis for "paired pulse facilitation" (PPF), put forward by Katz and Miledi (1968). In this scheme, elevated [Ca^{2+}] following a first stimulus would lead to an increased p during a second pulse. The decay of this PPF with increasing time interval between the two pulses would reflect the decay of residual [Ca^{2+}]. Many objections have been raised against this view, mostly based on the argument that a small increment in basal [Ca^{2+}] would not really matter a lot if added to the microdomain peak [Ca^{2+}], which is one to two orders of magnitude higher than residual [Ca^{2+}] (see Zucker and Regehr, 2002, for a review on this debate). Also, it was found that calcium currents facilitate and inactivate with time courses similar to those of facilitation and depression of synaptic transmission (Borst and Sakmann, 1998; Forsythe et al., 1998; Cuttle et al., 1998; Inchauspe et al., 2004; Xu and Wu, 2005; Ishikawa et al., 2005; Mochida et al., 2008). However, quantitative comparisons between dynamic changes in currents and EPSCs came to the conclusion that changes in release cannot be fully attributed to changes in Ca^{2+} current under all recording conditions (Kreitzer and Regehr, 2000; Felmy et al., 2003; but see Xu and Wu, 2005). Also, it must be considered that an increase in p will be obscured by a concomitant decrease in pool size when measuring EPSCs in phasic synapses (Rozov et al. 2001; Zucker, 1973)—in contrast to the case of tonic synapses, in which vesicle consumption is compensated by an enhanced recruitment. A quantitative study in the calyx of Held (Hosoi et al., 2007), in which an attempt was made to separate changes in pool size from those in p, came to the conclusion that, during a 100 Hz stimulus train, p increases during the initial 5–10 APs by a factor of five, while pool size decreases by more than a factor of ten, leading to net depression (see below). A recent study on transgenic mice (Müller et al., 2007) in which the slow Ca^{2+}-binder parvalbumin was ablated showed characteristic changes in the decay of [Ca^{2+}] transients following single APs. These were paralleled by changes in PPF, strongly suggesting that a large part of PPF is directly linked to the global [Ca^{2+}] signal in the nerve terminal. Nevertheless, a tight correlation between Ca^{2+}-current dynamics and PPF has been shown (Inchauspe et al., 2004; Ishikawa et al., 2005). The argument that the increment in basal [Ca^{2+}] is too small to explain PPF can be countered by the possibility of nonlinear summation of basal and microdomain [Ca^{2+}], which may happen in the presence of fast saturable [Ca^{2+}] buffers (Neher, 1998). Such a mechanism has been found in hippocampal mossy fiber synapse (Blatow et al., 2003).

These examples and debates about PPF illustrate that EPSCs represent a complex interplay between mechanisms, which control vesicle exocytosis and the availability of releasable vesicles. Under conditions when initial release probability is low, only a small fraction of the vesicle pool is consumed during a first stimulus. Thus, an increase in p can manifest itself during a second stimulus. If, however, a larger fraction of the pool is consumed during a first AP, the increase in p may be superseded by the fact that only a reduced pool is left over—leading to net depression. Kinetic models, which incorporate both facilitation and consumption of vesicles, can nicely reproduce the behavior of various type of synapses (Dittman et al. [2000] for climbing fiber and parallel fiber synapses in the cerebellum as well as

Schaffer collateral to CA1 synapses of hippocampus cells; Varela et al. [1997] for cortical synapses). However, definite answers about the interplay between facilitation and depression may be possible only in synapses, in which these aspects can be teased apart experimentally, and if postsynaptic contributions are allowed for (Foster et al., 2002). Neglect of postsynaptic effects can readily lead to incorrect conclusions about pool dynamics (Weis et al., 1999).

The Calyx of Held

One preparation in which a distinction between changes in vesicle pools and intrinsic release properties is possible is the calyx of Held. This structure is the presynaptic terminal of a synapse in the auditory pathway located in the medium nucleus of the trapezoid body (MNTB) in the brainstem. Forsythe (1994) showed that the cup- or calyx-shaped terminal can be voltage clamped using the tight-seal whole-cell variant of the patch-clamp technique (Hamill et al., 1981). Borst et al. (1995) showed that, indeed, simultaneous dual voltage clamp of the pre- and postsynaptic compartments is possible and that the presynaptic terminal can be loaded via the patch pipette with Ca^{2+} chelators and indicator dyes (Borst and Sakmann, 1996; Helmchen et al., 1997). Presynaptic [Ca^{2+}] can be measured fluorimetrically (Helmchen et al., 1997), and release can be triggered by flash photolysis from caged Ca^{2+} (Bollmann et al., 2000; Schneggenburger and Neher, 2000). Thus, the synapse has many attributes of the "perfect" synapse for a biophysical dissection of neurotransmitter release. However, it does have its caveats. The age of animals at which such measurements are best performed is from postnatal day 8 to 10 (in rats), at which time the synaptic properties are not yet fully matured (Taschenberger and von Gersdorff, 2000; Iwasaki and Takahashi, 2001). At this age, there is significant accumulation of "spillover glutamate" in the synaptic cleft during strong stimulation, resulting in postsynaptic desensitization. This must be allowed for computationally (by a special deconvolution technique) and pharmacologically (using cyclothiazide and kynurenic acid) if one wants to use the postsynaptic current as an assay of release (Neher and Sakaba, 2001a, 2001b). Alternatively, capacitance measurement (Neher and Marty, 1982) can be used for monitoring exocytosis (Sun and Wu, 2001). In addition, the calyx of Held shows another complication, a distinctly heterogeneous vesicle pool.

Heterogeneity of vesicles with respect to their release probability has been described in several types of synapses (Walmsley et al., 1988; Hessler et al., 1993; Rosenmund et al., 1993). In the calyx of Held this was first shown by Wu and Borst (1999), who observed strong synaptic depression after a 10 ms depolarization of the presynaptic terminal and suggested that the remaining release was due to "rapidly replenished but reluctant" vesicles. Sakaba and Neher (2001a) studied this phenomenon in detail and found that with inclusion of some EGTA (0.5 mM) in the presynaptic pipette the response to a 50 ms depolarization can be reproducibly split into a fast component of release with a time constant of 2–3 ms and a second one about ten times slower. Since Ca^{2+} current is approximately constant during this interval, they argued that this result could best be explained by two vesicle populations, each harboring about 1500 vesicles, with mean lifetimes (until exocytosis after the onset of depolarization) of about 3 ms and 30 ms, respectively. Below, we will discuss the question of why one type of vesicles reacts quickly while the other one reacts slowly.

Since a calyx terminal has about 500 to 600 active zones (Sätzler et al., 2002), a single active zone will harbor on average about three fast vesicles and three slow ones. With a maximal stimulus at elevated extracellular [Ca^{2+}], 85% of these vesicles can be released within 5 ms (Sakaba and Neher, 2001a). Furthermore, it was found that, after a depleting stimulus, slowly releasing vesicles recover very rapidly (Wu and Borst, 1999; Sakaba and Neher, 2001b), while fast vesicles recover slowly. Also, it turned out that the slow recovery of fast vesicles is accelerated when global [Ca^{2+}] rises in the terminal (e.g., during strong stimulation or after infusing Ca^{2+}-EGTA mixtures). An important role of calmodulin could be assigned to this Ca^{2+}-dependent process, since calmodulin blockers prevented the acceleration of recovery (Sakaba and Neher, 2001b) and it was shown that a sufficient level of cAMP must prevail for this acceleration to take place (Sakaba and Neher, 2003b). In contrast, recovery of the slow vesicle population was always fast (<100 ms) and was not influenced by calmodulin blockers and manipulations regarding cAMP. The only perturbations that slowed down recovery of the slowly releasing vesicles were depletion of ATP and disruption of the cytoskeleton by latrunculin (Sakaba and Neher, 2003a). Alternative interpretations for slow release components have been offered, such as adaptation of the release apparatus (Hsu et al., 1996, Wölfel et al., 2007) or rapid recruitment of vesicles (Kushmerick et al. 2006). Indeed, the separation between slow release and rapid recruitment, which is immediately followed by release during an ongoing stimulus, is not straightforward. During long depolarizations, release rates at the calyx of Held settle to a plateau of about 10–15 vesicles/ms, which further decays with half-times of 50–100 ms (Neher and Sakaba, 2001b). This is less than an order of magnitude slower than the slow component and therefore not readily isolated. However, exposure of calyces with latrunculin and infusion of ATP-γ-S retarded recovery of release significantly while leaving the time course of release itself (both fast and slow components) almost unchanged. This would not be expected if the slow component were due to rapid recruitment. Slow components of release have been described, next to the calyx, in mossy fiber boutons (Hallermann et al., 2003) and ribbon-type synapses (Mennerick and Matthews, 1996; Moser and Beutner, 2000) but are not very prominent at inhibitory synapses (Sakaba, 2008). Physiologically more important for AP-induced (synchronous) release are the rapidly releasing vesicles (Sakaba, 2006), while the slowly releasing ones may contribute to asynchronous release during and after high-frequency firing. Therefore, most of the conclusions to be discussed below do not hinge on an accurate separation of slow release and rapid recruitment. Nevertheless, its kinetic characterization offers important insights with regard to molecular mechanisms (see below).

The Ca^{2+} dependence of recruitment of fast-releasing vesicles after a depleting stimulus was studied in a quantitative manner (Hosoi et al., 2007). Various manipulations with global intraterminal [Ca^{2+}] showed that there is a linear relationship between the recruitment rate of fast-releasing vesicles and [Ca^{2+}] with a basal value of 0.1 pools/s at zero [Ca^{2+}] (i.e., the pool refills with a time constant of 10 s). The slope of this relationship has been found to

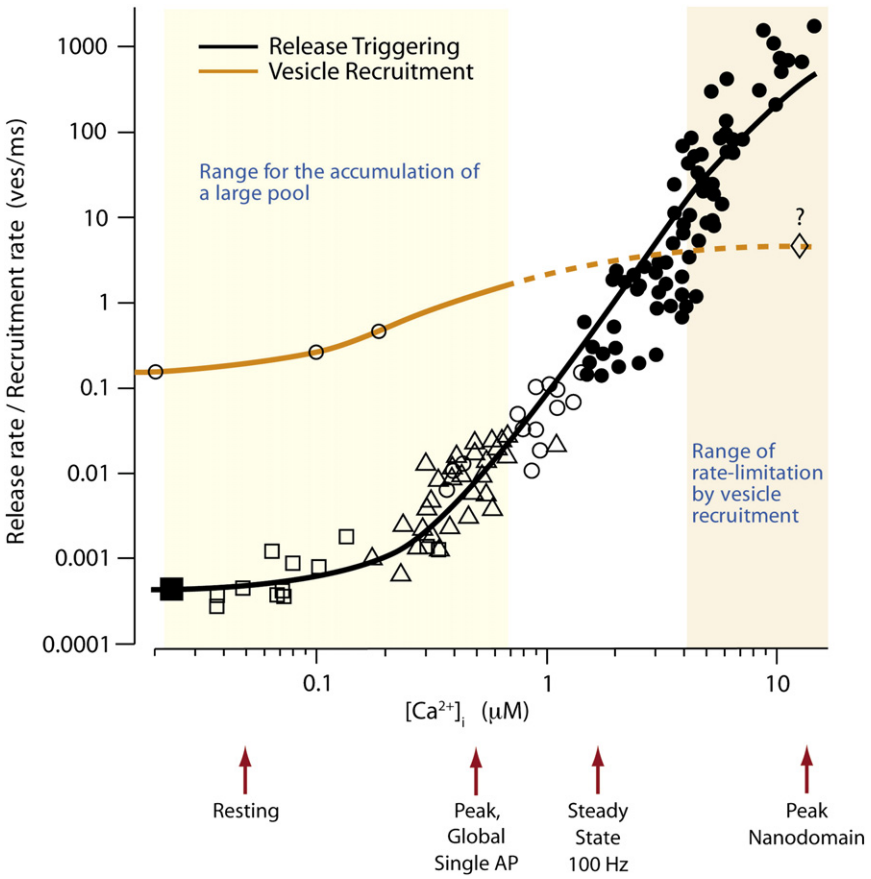

Figure 1. Comparing Ca^{2+} Dependence of Release-Triggering with that of Vesicle Recruitment

The intracellular dose-response curve for Ca^{2+} action in vesicle release at the calyx of Held (— and symbols; taken from Lou et al., 2005 [reprinted by permission from Macmillan Publishers Ltd: *Nature*, copyright 2005) is shown together with the Ca^{2+} dependence of vesicle recruitment (yellow line) according to Hosoi et al. (2007). Ca^{2+} dependence of vesicle recruitment cannot be measured accurately at higher $[Ca^{2+}]$ because of ongoing release of synaptic vesicles, and therefore, a dotted yellow line was used as a prediction for a Michaels-Menten type Ca^{2+} dependence. Colored regions of the graph represent the ranges of $[Ca^{2+}]$ in which either the recruitment rate is much larger than release (left side) or the opposite holds true (right side). The slope of the dose-response curve approaches 4 in its steepest portion, while that of vesicle recruitment is ≤ 1. Values are given for the calyx of Held. At inhibitory synapses (Sakaba, 2008), the steepness parameters are similar (Table 1); however, the crossover of the two curves occurs already at 1 μM. Resting $[Ca^{2+}]$ level (50 nM), peak global $[Ca^{2+}]$ increase after action potential (500 nM; Müller et al., 2007), steady-state $[Ca^{2+}]$ during a 100 Hz stimulus train (1.5 μM; Hosoi et al., 2007), as well as peak nanodomain-$[Ca^{2+}]$ are indicated by arrows.

be about 1/(μMs), meaning that at 1 μM $[Ca^{2+}]$ the refilling rate is 1.1 pools/s or else the time constant is 900 ms. Applying 100 Hz stimulation, it was found that intraterminal $[Ca^{2+}]$ rose to ~1.5 μM during such a tetanus, and, consequently, the recruitment of vesicles was accelerated many-fold relative to the recruitment at resting $[Ca^{2+}]$. Therefore, steady-state EPSCs during stimulation were an order of magnitude higher than they would be if acceleration of recruitment would not take place. In experiments under the influence of calmodulin blockers, substantial reduction of steady-state release and deeper short-term depression during 100 Hz stimulus trains could be confirmed.

Figure 1 summarizes two of the $[Ca^{2+}]$ effects discussed so far. It shows the intracellular dose-response curve for $[Ca^{2+}]$ triggering, as obtained from caged-$[Ca^{2+}]$ experiments (Bollmann et al., 2000; Schneggenburger and Neher, 2000; Lou et al., 2005), together with the $[Ca^{2+}]$ dependence of vesicle recruitment. Here, a qualification to the above discussion of the latter relationship is required. The linear dependence of recruitment rate upon global $[Ca^{2+}]$ was shown for $[Ca^{2+}]$ values between 0 and ≈1 μM (Hosoi et al., 2007). It cannot be expected that the acceleration of recruitment will continue indefinitely when raising $[Ca^{2+}]$. An upper limit for recruitment of about 10 vesicles/ms can be defined by the steady-state release, observed during a strong stimulus at some 50–100 ms after its onset (Neher and Sakaba, 2001b). Therefore, the curve drawn in Figure 1 for vesicle recruitment is a Michaelis-Menten relationship (as originally suggested by Dittman and Regehr, 1998) with an initial slope of 1 pool/(μMs), an offset of 0.1 pools/s, and a maximum recruitment rate of 5 vesicles/ms or 3.3 pools/s—assuming that 50% of the vesicles are recruited to a fast pool of 1500 vesicles. In spite of the uncertainty about recruitment at higher $[Ca^{2+}]$, Figure 1 shows clearly that there is a regime of $[Ca^{2+}]$ values (from zero to about 500 nM) in which the recruitment rate is much higher than the release rate. This is the range within which a large fraction of the pool of releasable vesicles remains available for release and within which release rates are controlled by the Ca^{2+}-triggering mechanism. In contrast, at $[Ca^{2+}]$ values above 5 μM, release rates are higher than those for recruitment. Therefore, the pool of vesicles will be depleted when $[Ca^{2+}]$ is maintained at such high values, and the rate-limiting step for overall release is vesicle recruitment. The two curves intersect because release follows a high-power function of $[Ca^{2+}]$ while recruitment is linear in $[Ca^{2+}]$ or rather saturating. Dose-response curves measured during slow variations in $[Ca^{2+}]$ (such as obtained with K^+ depolarization or calcimycin) are expected to follow the "release triggering" curve up to ~1–2 μM and to merge with the "vesicle recruitment" curve for higher $[Ca^{2+}]$. The "apparent Ca^{2+} affinity" of such a curve would be ~2–3 μM and the dynamic range at most four orders of magnitude, instead of seven.

Two other features of Ca^{2+}-dependent vesicle recruitment should be mentioned, which are consequences of parameters characterizing recruitment and $[Ca^{2+}]$ dynamics at the calyx of Held.

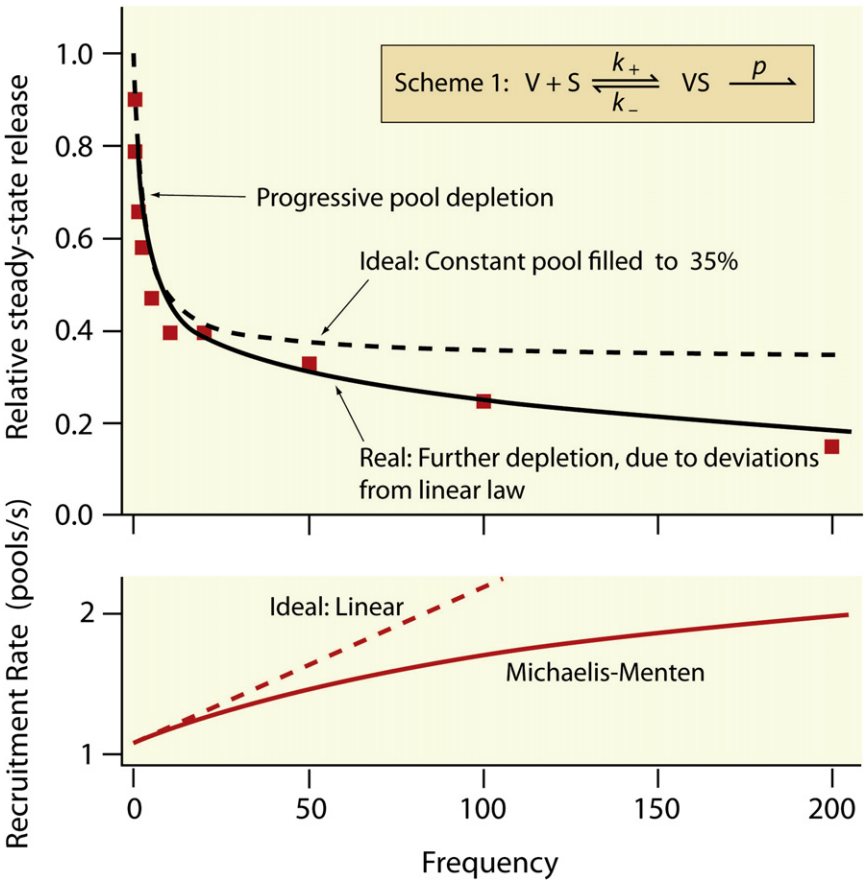

Figure 2. Short-Term Depression with Ca^{2+}-Dependent Recruitment of Vesicles; Expectations of a Simplified Model

The upper panel shows quantal content of EPSCs at steady state during high-frequency stimulation, relative to that at stimulus onset, plotted against frequency, *f*. Dotted line (···): expectations for the scheme of pool depletion (scheme 1) assuming a strictly linear recruitment rate, which is strictly linear with $[Ca^{2+}]$ with slope *k* and a linear dependence of steady-state $[Ca^{2+}]$ on frequency. Straight line (—): same model; however, assuming a Michaelis-Menten-type $[Ca^{2+}]$ dependence of the recruitment rate, with the same initial slope (1.5 pools/(μMs) and a K_M of 2.13 μM. Facilitation has been neglected in this calculation. The slope S_{Ca} of steady-state $[Ca^{2+}]$ as a function of *f* was assumed to be 15 nM/Hz. Data points are approximate values of relative quantal content at the calyx of Held, kindly provided by Holger Taschenberger. The steady-state filling of the pool for the high-frequency limit in the ideally linear case (35%) and the high-frequency limit is calculated from the expression $k \times s_{ca}/(k \times s_{ca} + p)$, where *p* is the probability of a given vesicle to be released during an action potential, assumed to be 0.043. k was assumed to be 1.5 pools/(μM × s) and s_{Ca} was 0.015 μM × s. (Lower panel) Recruitment rates for both the linear (—) and the Michaelis Menten model (—). Note that the release rate (upper panel) drops steeply as long as the recruitment rate is close to its basal value (≈0.1). However, for frequencies beyond 10 Hz, the linear rise dominates, keeping the release constant.

- $[Ca^{2+}]$-dependent recruitment is hard to observe *after* a train of stimuli. The decay of the global $[Ca^{2+}]$ elevation is so fast after a short burst of stimuli (time constant at 30–100 ms, Müller et al., 2007) that little recovery of depression is expected before basal $[Ca^{2+}]$ levels are reached (for a decay from 1.5 μM and 50 ms time constant, the pool will recover by 7.5%). The majority of recovery will occur at resting levels. Correspondingly, Wang and Kaczmarek (1998), who first described Ca^{2+}-dependent recovery at the calyx of Held, had to resort to the strongest possible stimulation to demonstrate this effect. Nevertheless, Ca^{2+} enhancement of recruitment is very powerful *during* a train of stimuli.
- $[Ca^{2+}]$-dependent recovery is well described by global $[Ca^{2+}]$, although any "sensor" for this mechanism is expected to be near the membrane and to "sense" microdomain $[Ca^{2+}]$. Again, this is a consequence of rapid $[Ca^{2+}]$ dynamics, since a microdomain within Compared to that, the global elevation of $[Ca^{2+}]$ in the interspike interval (1.5 μM at 100 Hz) will refill about 1.2% during the remaining 8 ms.which $[Ca^{2+}]$ reaches high levels, but for only 1 or 2 ms, will refill not more than 0.66% of the empty sites (at the maximum rate of 3.3 pools/s). Thus, the majority of refilling would not occur in the absence of global $[Ca^{2+}]$ elevations. These considerations relate to the recording conditions of Hosoi et al. (2007), where either EGTA and/or indicator dye was present in the presynaptic terminal. In unperturbed terminals, diffusion may be slower (Scheuss et al., 2007; Müller et al., 2007; Gabso et al., 1997), which will give some more weight to the contributions of local $[Ca^{2+}]$ elevations.

What would be the physiological consequences of a linearly rising rate of vesicle recruitment within the framework of a model of vesicles recruitment and consumption? Figure 2 summarizes some of the expectations of a simple model that captures a number of interesting features of short-term depression but, for simplicity, neglects influences of Ca^{2+} current dynamics. Quite remarkably, the prediction is that for calyx of Held parameters the quantal content of EPSCs during trains of stimuli should be independent of stimulation frequency (*f*) above 10 Hz—if it is assumed that steady-state $[Ca^{2+}]$ rises linearly with *f* in this same frequency range. This is because quantal content in this regime is limited by vesicle recruitment, and the latter rate rises linearly with *f* (due to linearly rising $[Ca^{2+}]$), such that a constant number of vesicles is recruited during an interstimulus interval, which varies with 1/*f* (see Figure 2 for illustration and numbers). Counterintuitively, in such a scenario the level of depression would not necessarily increase with an increase in release in an experiment in which release is enhanced by an increase in Ca^{2+} influx. An increased recruitment may compensate for increased consumption of vesicles. For example, at phasic synapses of the

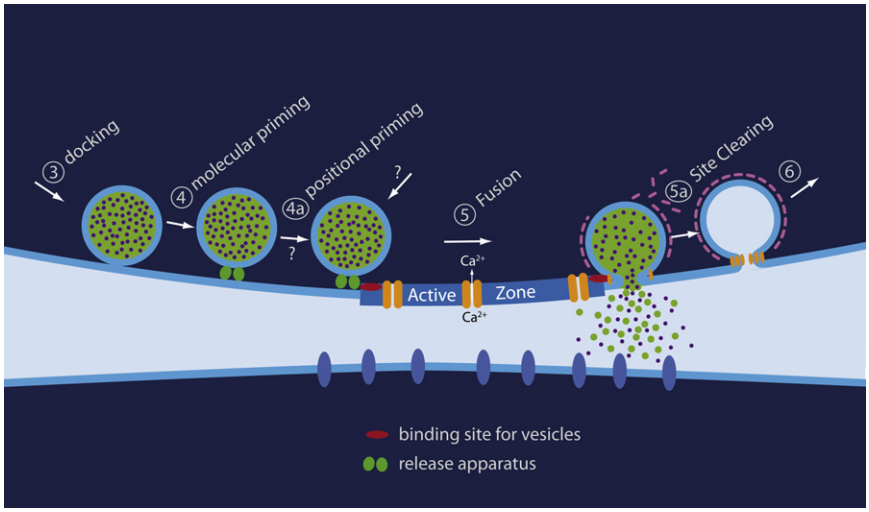

Figure 3. Part of the 'Vesicle Cycle' of Exo- and Endocytosis

For simplicity, the case of "kiss and run" exo-endocytosis is depicted on the right side. However, the argument about rate limitation of the site-clearing step 5a would also hold for classical endocytosis, if it is assumed that the interaction between an active zone component and some component of the vesicle or release machinery has to be reversed before another vesicle can dock.

Drosophila neuromuscular junction, increased release at higher [Ca^{2+}] did not change the level of depression, but this finding was taken as evidence against a vesicle-depletion mechanism (Wu et al., 2005).

The linear laws assumed in the above paragraph have been confirmed only over limited ranges of *f* (Hosoi et al., 2007; Helmchen et al., 1997). In reality, they are not expected to prevail at frequencies as high as hundreds of Hertz. Correspondingly, one observes a gradual drop in steady-state EPSC amplitudes at high *f* instead of a plateau. It is not clear, presently, whether sublinear vesicle recruitment, sublinear [Ca^{2+}] increase, or other influences are responsible for the drop. However, in cortical neurons, Dittman and Regehr (1998) could well describe the frequency dependence of release by a Michaelis-Menten-type dependence of recruitment (see also Figure 2).

In summary, we conclude that a rate-limiting step for AP-induced release during stimulation above 10 Hz is the recruitment of vesicles to the fast-releasing pool. This step is highly modulated by [Ca^{2+}], cAMP, and GTP-binding proteins (Takahashi et al., 2000) and is a major mechanism of short-term depression. Contributions of other mechanisms, such as postsynaptic receptor desensitization (Koike-Tani et al., 2008) and Ca^{2+} current inactivation (Forsythe et al., 1998; Xu and Wu, 2005) have been demonstrated, with some disagreement between laboratories about the magnitude of such effects. In our hands, and with the stimulation protocols of Hosoi et al. (2007), Ca^{2+} current inactivation is minor and overcompensated by Ca^{2+}-dependnet facilitation. A quantitative separation of contributions under physiological conditions is most relevant in the calyx of Held, since auditory nerve fibers display tonic activity in the range 0.15–110 Hz, even in complete silence (Kopp-Scheinpflug et al., 2003; Hermann et al., 2007). Mechanistically, one of the most pressing questions, currently, regards the molecular nature of the rate-limiting step discussed above.

'Molecular' versus 'Positional Priming'

In principle, one can consider three mechanisms for explaining why some vesicles release rapidly and others slowly. The simplest reason may be that they differ in their release apparatus—some vesicles responding more rapidly to a given [Ca^{2+}] than others. Indeed, heterogeneity in intrinsic Ca^{2+} sensitivity has been observed in a caged-Ca^{2+} study at the calyx of Held (Wölfel et al., 2007). It may well contribute to the heterogeneity observed during depolarizing stimuli. Second, Ca^{2+} current inactivation may lead to a decrease in release probability during trains of stimuli and during long-lasting depolarizations (see references quoted above). Third, or in addition, there may be a difference regarding the positioning of "molecularly primed" vesicles with respect to nearby Ca^{2+} channels. In other words, slowly releasing vesicles may be perfectly release competent with respect to their intrinsic Ca^{2+} sensitivity, but located too far away from Ca^{2+} channels (which are known to be clustered at active zones; Roberts et al., 1990; Adler et al., 1991; Llinas et al., 1992; Haydon et al., 1994; Khanna et al., 2007; Bucurenciu et al., 2008; Kittel et al., 2006). They would not sense the [Ca^{2+}] of the nanodomain and not get released during very short episodes of [Ca^{2+}] influx. A recent study tested this hypothesis by measuring, via caged-Ca^{2+} stimulation, the intrinsic [Ca^{2+}] sensitivity of those vesicles remaining after the rapidly releasing pool had been depleted by a 10 ms depolarization (Wadel et al., 2007). It turned out that these vesicles, which are released by depolarization several-fold slower than the fast ones, are almost as sensitive to [Ca^{2+}] as under control conditions. Their dose-response curve was downshifted by at most a factor of two. Based on these data, it was concluded that a major reason why reluctant vesicles are slowed down (up to a factor of ten) must be a longer distance to the nearest Ca^{2+} channels, with intrinsic heterogeneity and Ca^{2+} current inactivation not playing major roles under the conditions of this experiment.

This leaves one important question to answer: what is the sequence of events that leads to a situation in which about half of the vesicles are slow and the other half are fast? Figure 3 shows two scenarios that may be considered. On the left side of the cartoon, we postulate that a step of molecular priming (the buildup of the release machinery, step 4) is followed by a lateral "mooring" at the active zone (step 4a). We call this step "positional priming." This step will convert a slowly releasing vesicle into a rapidly releasing one. We have to postulate that this step is rate limiting, since we know that step 4, the recruitment of slowly releasing vesicles, is fast. Also, we have to postulate that this step is strongly modulated by Ca^{2+}. Molecular mechanisms mediating positional priming may include interactions of the release machinery, via Munc13, with active zone components, like Rim

(Weimer et al., 2006; Dulubova et al., 2005; Andrews-Zwilling et al., 2006) or Cast (Kittel et al., 2006). Direct interactions with Ca^{2+} channels are unlikely on biophysical grounds, since the distance to nearest Ca^{2+} channels is estimated to be 30–60 nm (Meinrenken et al., 2002), although it may become smaller during development (Fedchyshyn and Wang, 2005). As in the case of molecular priming, Ca^{2+} sensitivity may be conferred to this step by the calmodulin interaction of Munc13 (Junge et al., 2004).

However, once it is accepted that there is a limited number of special mooring or binding sites on the active zone, there is an alternative explanation for the slowness of recruitment of vesicles to such sites. This explanation starts with the question about the fate of such sites after a fusion event. Obviously, they must have interacted with components of the vesicle or the release machinery, and this interaction must dissociate before another vesicle can dock. It is easily conceivable that this reaction is the rate-limiting step for recruitment of fast-releasing vesicles, as is implied in the models of Dittman and Regehr (1998). If so, it would probably be a step in between neurotransmitter release and the endocytosis of vesicular components, as shown diagrammatically in Figure 3 (step 5a: "site-clearing").

There is some evidence for such a step in the literature, such as the transfer of vesicular/exocytosis components from sites of release to special zones of endocytosis (Roos and Kelly, 1999; Teng and Wilkinson, 2000; Wienisch and Klingauf, 2006). Rate-limitation of vesicle recruitment may, therefore, be a consequence of a delay in reprocessing of release sites or else a "traffic jam" in endocytosis. The expected phenotype for interference with this step is characterized by a normal amplitude of stand-alone EPSCs and an enhanced depression. Usually, such enhanced depression is attributed to decreased availability of vesicles for docking (step 3 in Figure 3) due to reduced recycling of vesicles. However, such depression sets in within a few stimuli (i.e., within 10 s of milliseconds), which is orders of magnitude too fast for reuse or "repriming" of vesicles (Betz and Wu, 1995; Nicholson-Tomishima and Ryan, 2004; Li et al., 2005). Following Kawasaki et al. (2000), we may propose that instead site clearance and repriming of sites is the rate-limiting step. Site clearance would have to happen at basal $[Ca^{2+}]$ within 5–10 s, and it would be accelerated many-fold by elevated $[Ca^{2+}]$ during trains of stimuli. Rapid forms of endocytosis, such as "kiss and run" endocytosis (Verstreken et al., 2002; Klyachko and Jackson 2002; He et al., 2006; Harata et al., 2006; Kavalali, 2007), have received much attention lately. They may be conceived as site-clearing steps in the sense of our discussion. However, we would like to point out that block of endocytosis leads to a large reduction in the amplitude of stand-alone EPSCs (Dickman et al., 2005; Ferguson et al., 2007), which quite likely reflects a reduction in the reserve pool of synaptic vesicles. It will be an interesting task to differentiate between endocytosis itself and any steps between exo- and endocytosis.

Molecular Outlook

The conclusions drawn here are based mainly on a biophysical analysis of phenomena that are well-known in synaptic physiology. Putting them into quantitative terms, which is possible for the calyx of Held, we arrive at interpretations of molecular perturbations that are quite different from the usual ones. We postulate a robust release machinery, interacting with a Ca^{2+} sensor, which increases release rate 10^7-fold when fully saturated with $[Ca^{2+}]$. In other words, Ca^{2+} binding can reduce the energy of activation by about 16 kT. Basic release is increased 5-fold by phorbol ester, or else activation of a phorbol ester site (either through Munc13 or through PKC) can contribute 1.6 kT to this energy. Quantitative analysis further suggests that one should make a distinction between molecular priming (involving the SNARE proteins, Munc18, Munc13, complexin,…) and positional priming (allowing primed vesicles to be near Ca^{2+} channels). Both are Ca^{2+} dependent. However, Ca^{2+} dependence of molecular priming may be physiologically rate limiting only in "tonic synapses" and in neuroendocrine cells, but not in "phasic synapses." We point out that positional priming can be a major rate limitation during high-frequency activity in phasic synapses. If so, spatial relationships and dynamic reorganizations of ultrastructural components turn out to be more important than generally assumed. In this sense, research on presynaptic aspects can be expected to recapitulate mechanisms of dynamic ultrastructual reorganization, which have captivated postsynaptic research over the last 10–20 years.

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