Commentary

Synaptotagmin Function Illuminated

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Neurotransmitter release occurs by exocytosis of synaptic vesicles triggered by Ca^{2+} influx through voltage-gated Ca^{2+} channels. It was found many years ago that release shows a steep dependence on extracellular $[Ca^{2+}]$ and increases approximately with the fourth power of $[Ca^{2+}]$ (Dodge and Rahamimoff, 1967). It was proposed that Ca^{2+} entering through voltage-gated Ca^{2+} channels binds to intracellular Ca^{2+} receptors, which cooperatively control the release of a vesicle (Katz and Miledi, 1967).

The kinetics of exocytosis can be monitored precisely and with high time resolution by whole-cell patch clamp capacitance measurements (Neher and Marty, 1982; Lindau and Neher, 1988) because the increase in membrane area due to vesicle fusion leads to a proportional increase in membrane capacitance. Such capacitance experiments can be conveniently performed in endocrine cells due to their simple equivalent circuit and revealed evidence that a small fraction of their secretory vesicles can be released very rapidly while many more are released much more slowly (Augustine and Neher, 1992; Lindau et al., 1992). However, when transmitter release is triggered by depolarization, $[Ca^{2+}]$ at the site of release is difficult to estimate because it depends on the rate at which Ca^{2+} ions move through the channels, as well as the distances between calcium channels and releasable vesicles due to intracellular $[Ca^{2+}]$ gradients (Roberts et al., 1990). Thus, this approach makes it difficult to determine precisely the relation between transmitter release rates and $[Ca^{2+}]$ because $[Ca^{2+}]$ at the sites of exocytosis is uncertain and furthermore time dependent.

These difficulties were overcome by using release of caged calcium from a photolabile Ca^{2+} chelator, which provides a rapid and uniform stepwise increase of intracellular $[Ca^{2+}]$, while exocytosis was monitored by capacitance measurements (Neher and Zucker, 1993; Thomas et al., 1993; Heinemann et al., 1994). This method showed that following a step increase of intracellular $[Ca^{2+}]$, exocytosis begins with a rapid exocytotic burst on the time scale of milliseconds followed by a slower, more sustained phase on the time scale of seconds. The exocytotic burst itself has a double exponential time course and its onset shows a slight delay relative to the $[Ca^{2+}]$ increase.

Extensive detailed studies have led to an interpretation of the exocytotic burst in terms of two vesicle pools, named the readily releasable pool (RRP) and the slowly releasable pool (SRP), that underlie the two exponential components of the exocytotic burst (Voets et al., 1999). The time course of release from the RRP and SRP are steeply $[Ca^{2+}]$ dependent, but their amplitudes are not. The $[Ca^{2+}]$ dependence of the amount of transmitter release in response to action potentials or brief depolarizations is thus a consequence of the $[Ca^{2+}]$ dependence of the release kinetics, which converts to a $[Ca^{2+}]$ dependence of the release amplitude when the intracellular $[Ca^{2+}]$ is elevated transiently (Voets et al., 1999). In this model, vesicles from both pools are released following binding of at least three Ca^{2+} ions, albeit at different rates (Voets, 2000). The affinity for the individual Ca^{2+} binding steps was estimated to be $\sim$10 μM for both pools but the Ca^{2+} binding and unbinding rates are $\sim$10 times slower for the SRP compared with the RRP giving rise to the difference in time course in response to a step change of $[Ca^{2+}]$ (Voets, 2000).

What is the mechanism by which Ca^{2+} triggers exocytosis of secretory vesicles? The vesicle membrane protein synaptotagmin 1 (for review see Chapman, 2002) has received much attention as a potential candidate for the Ca^{2+} receptor, or Ca^{2+} sensor, controlling synaptic vesicle exocytosis. The initial hint came from the discovery that this protein contains two copies of a repeat homologous to the regulatory C2 domain of calmodulin (Perin et al., 1990). These domains named C2A and C2B are in the cytosolic part of synaptotagmin 1, which is anchored in the vesicle membrane via a single transmembrane domain. Synaptotagmin 1 binds multiple Ca^{2+} ions involving its C2A and C2B domains (for review see Chapman, 2002).

The role of synaptotagmin as a Ca^{2+} sensor mediating neurosecretion has been a matter of controversy and confusion. It was originally proposed that synaptotagmin's C2A domain alone is responsible for Ca^{2+} binding (Davletov and Sudhof, 1993), whereas C2B was thought to mediate interactions with phospholipids, SNARE proteins, and Ca channels. This view was challenged by the finding that a mutation in C2A, which dramatically reduces its Ca^{2+} affinity, had no major effect on transmit-

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ter release (Fernandez-Chacon et al., 2002; Robinson et al., 2002). It has now become evident that Ca^{2+}-dependent activation of synaptotagmin 1 involves the double C2A/C2B domain (Fernandez-Chacon et al., 2001; Bai et al., 2002). The amazing thing about the Ca affinity of synaptotagmin is that the marked change in the Ca^{2+} affinity of the isolated C2A-domain fragment has no functional effect on transmitter release when the mutation is expressed in full-length synaptotagmin in the cell. This discrepancy recently was explained by the finding that this mutation does not change the affinity of the C2A/C2B tandem domain fragment (Fernandez-Chacon et al., 2002). In contrast, the mutation R233Q mutation produces a small but significant change in Ca^{2+} affinity of the C2A/C2B tandem domain, which is associated with a corresponding functional shift in Ca^{2+} sensitivity of transmitter release (Fernandez-Chacon et al., 2001, and this paper).

Disruption of the synaptotagmin 1 gene in mice abolished synchronous release in hippocampal neurons, but did not affect the Ca^{2+} dependence of the remaining asynchronous release (Geppert et al., 1994). Although suggestive, this result did not clearly indicate what the function of synaptotagmin might be. Other than being the calcium sensor for the rapid release phase, the results could also be explained if disruption of synaptotagmin 1 disrupts colocalization of vesicles with calcium channels because synaptotagmin 1 also interacts with Ca channels (Charvin et al., 1997; Kim and Catterall, 1997).

These ambiguities could be eliminated using photorelease of caged Ca^{2+} such that $[Ca^{2+}]$ is homogeneously elevated bypassing involvement of Ca^{2+} channels. Using this approach it was shown that in chromaffin cells from synaptotagmin knock-out mice the fastest release phase of vesicles in the RRP was absent, while the slower components (SRP and sustained phase) were unaffected. Accordingly, exocytotic responses to brief depolarizations were markedly reduced. In experiments with caged Ca^{2+} no Ca^{2+} gradients occur, so these results cannot be explained by disrupting colocalization of synaptic vesicles with Ca channels. Rather, it can be concluded that synaptotagmin 1 is required for the fast component of release, being either a prerequisite for existence of the RRP or the Ca^{2+} sensor governing release from this pool.

To distinguish between these two possibilities, Sørensen et al. (2003, in this issue) made use of the synaptotagmin 1 mutant R233Q that has a twofold lower Ca^{2+} affinity than wild-type synaptotagmin 1 (Fernandez-Chacon et al., 2001). If synaptotagmin 1 were required for the existence of the RRP, then the R233Q mutant would be expected to be fully functional or to reduce the relative amplitude of release coming from the RRP. In contrast, if synaptotagmin 1 were the Ca^{2+} sensor for release from the RRP, then a shift of the $[Ca^{2+}]$ dependence of the release kinetics from the RRP would be expected. The experimental results are: First, in chromaffin cells expressing the R233Q mutant both components of the exocytotic burst are present and the relative size of the SRP vs. RRP is unchanged. Secondly, at similar $[Ca^{2+}]$ both the delay and the time constant of the fast burst component, representing release from the RRP, are increased about twofold compared with cells expressing wild-type synaptotagmin 1 (Sørensen et al., 2003, in this issue). These results provide strong evidence that synaptotagmin 1 is indeed the calcium sensor for release from the RRP rather than a molecule determining the pool size. The main finding is very clear and the conclusion is straightforward.

In accordance with the flash results, experiments where $[Ca^{2+}]$ was gradually increased by slow photolysis ($[Ca^{2+}]$ ramp) the secretory response in R233Q expressing cells is shifted to later times corresponding to higher Ca^{2+} concentrations. Using the previously developed model to analyze the data, Sørensen et al. (2003) find that in cells expressing the R233Q mutant, the Ca^{2+} affinity of the Ca^{2+} sensor mediating release from the RRP is decreased twofold. Remarkably, the Ca^{2+} dependence of lipid binding of the cytosolic domain of synaptotagmin 1 shows the same shift in this synaptotagmin 1 mutant (Fernandez-Chacon et al., 2001). The analysis by Sørensen et al. (2003, in this issue) further reveals that the decrease in Ca^{2+} affinity comes from a correspondingly decreased on-rate of Ca^{2+} binding.

The R233Q mutation also affects release from the SRP. Although the authors consider the kinetic data for release from this pool to be less reliable, they suggest a similar shift of Ca^{2+} affinity. But, in the previously mentioned experiments with synaptotagmin 1 knock-out cells, the release from the RRP was absent while release from the SRP was unchanged. It appears that release from the SRP is normally mediated by another Ca^{2+} sensor, presumably another isoform of synaptotagmin with similar Ca^{2+} affinity but lower on- and off-rates for Ca^{2+} binding (Voets, 2000). This would be consistent with the old result that asynchronous release and its $[Ca^{2+}]$ dependence is unchanged when synaptotagmin 1 is disrupted (Geppert et al., 1994). If or how synaptotagmin 1 or the R233Q mutant is somehow affecting or participating in release from the SRP is a matter to be solved in further work.

An unexpected result is the increase in size of both the RRP and SRP by a factor of ~1.5–2 in cells expressing the mutant synaptotagmin 1 R233Q. One possibility would be that release at resting $[Ca^{2+}]$ is reduced in R233Q cells due to the reduced Ca^{2+} affinity. This could lead to an increase in the pool size at steady-state. The increase in the size of the SRP is comparable to the increase in the size of the RRP. At steady-state, however,

this could potentially result from the slow equilibrium between SRP and RRP and does not necessarily require a direct involvement of synaptotagmin 1 in the regulation of or release from the SRP.

Clearly, much has to be learned about the Ca^{2+} sensor mediating release from the SRP. The work of Sørensen et al. (2003) has provided very strong evidence that synaptotagmin 1 is the essential Ca^{2+} sensor involved in release from the RRP and the results are consistent with a mechanism where synaptotagmin 1 is the exclusive Ca^{2+} sensor for release from this pool. The similarities with Ca^{2+}-dependent lipid binding suggest that Ca^{2+} triggering of plasma membrane lipid binding may be involved in triggering fusion. However, other proteins such as SNAREs and complexins are presumably involved in Ca^{2+}-triggered fusion (for review see Jahn et al., 2003). The detailed molecular mechanism by which Ca^{2+} binding by synaptotagmin 1 leads to fusion pore formation is still far from clear. Sørensen et al. (2003, in this issue) did not observe detectable effects of the R233Q mutant on the lifetime of the initial fusion pore (foot duration). This could be explained if synaptotagmin 1 does not affect the fusion pore once it has initiated its formation. However, it was recently reported that overexpression of synaptotagmin 1 or 4 modulates fusion pore properties in PC12 cells (Wang et al., 2001). It is possible that the only change due to the R233Q mutation is the decreased Ca^{2+} affinity. Once it has bound Ca^{2+}, it may then be functionally indistinguishable from the wild-type protein. It thus remains an open question how synaptotagmin is mechanistically related to the fusion pore. The work of Sørensen et al. (2003) provides very strong evidence in support of synaptotagmin as the Ca^{2+} sensor for transmitter release. Much more work needs to be done to fully understand how Ca^{2+} binding by synaptotagmin is coupled to other proteins and fusion pore formation.

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