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Synaptic Vesicle Endocytosis

Calcium Works Overtime in the Nerve Terminal

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Abstract

The functions of Ca^{2+} are many and varied within cells, but in the nerve terminals of neurons it has had a very defined role. That is, the influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels stimulates neurotransmitter release by exocytosis. For years this was assumed to be the main role for Ca^{2+} in this specialized subcellular region. However recent studies have shown that Ca^{2+} also has multiple roles in synaptic-vesicle endocytosis. This review will present evidence for three Ca^{2+}-dependent and -independent steps; a high-affinity Ca^{2+}-dependent triggering step, a Ca^{2+}-independent maintenance phase, and a low-affinity Ca^{2+}-dependent inhibition step. How the control of endocytosis by Ca^{2+} might impact on different neuronal functions such as synaptic transmission, the nucleation of SV endocytosis, and the repair of damaged membrane is then discussed.

Index Entries: Calcium; endocytosis; exocytosis; synaptic vesicle; nerve terminal; calmodulin; calcineurin; dynamin; actin; kiss-and-run.

Introduction

On invasion of a nerve terminal by an action potential, voltage-dependent Ca^{2+} channels (VDCCs) are activated, which allow extracellular Ca^{2+} to enter nerve terminals down a large concentration gradient. Ca^{2+} influx triggers the fusion of neurotransmitter-containing synaptic vesicles (SVs) with the nerve-terminal plasma membrane (exocytosis). Neurotransmitter release is essential for synaptic communication and if the process is arrested, paralysis and death quickly follow *(1)*. With such an important role to play, it was assumed that the main task for Ca^{2+} in the SV-recycling pathway would be to stimulate exocytosis and neurotransmitter release. However there is now strong evidence that Ca^{2+} influx is employed in other aspects of the SV-recycling pathway such as endocytosis, which is the mechanism that retrieves SVs after exocytosis.

The role of Ca^{2+} in SV endocytosis has been debated for a number of years, mainly as a result of early studies that yielded apparently conflicting results. In these studies Ca^{2+} influx was proposed to either stimulate *(2,3)*, have no effect at all *(4,5)* or inhibit endocytosis *(6)* in different types of excitable cells. In central neurons, the consensus view was that SV endocytosis was a Ca^{2+}-independent process that was activated by the fusion of SVs with the plasma membrane. This model made good sense, because SV endocytosis was known to be intimately coupled to exocytosis to ensure that SVs were recycled reliably and quickly. There is now compelling evidence for an essential role for Ca^{2+} in endocytosis in neurons and other excitable cells however, and new models have been proposed that explain all of the early conflicting data. This review will discuss the evidence for multiple roles for Ca^{2+} in endocytosis and how it fits together in one unifying model. The relationship between this model and different aspects of neuronal function will then be discussed.

Does Ca^{2+} Influx Trigger Endocytosis?

SV endocytosis is so closely coupled to exocytosis in neurons that it was long assumed to be a Ca^{2+}-independent process that was activated by SV fusion. However, an essential role for Ca^{2+} influx in endocytosis has now been demonstrated in neurons as well as in other excitable cells. The first evidence of this essential role came from studies using black widow spider venom and the toxin derived from the venom, α-latrotoxin. A massive exocytosis of SVs was stimulated in either the presence or absence of extracellular Ca^{2+} when the venom or toxin was added to frog neuromuscular junction (NMJ) nerve terminals *(7)*. SV endocytosis could not be stimulated without extracellular Ca^{2+}, suggesting a requirement for Ca^{2+} influx *(2,3)*.

Subsequent investigations have confirmed that Ca^{2+} influx is essential for endocytosis in central neurons, secretory cells and NMJ nerve terminals. For example, in secretory cells, both compensatory endocytosis (which retrieves the same amount of membrane that was incorporated by exocytosis), and excess/rapid endocytosis (which retrieves more membrane with faster kinetics), were shown to be Ca^{2+}-dependent. These studies showed endocytosis was stimulated by increases in intracellular-free Ca^{2+} ($[Ca^{2+}]_i$; *8–13*), inhibited by chelation of $[Ca^{2+}]_i$ *(14)*, or inhibited by substitution of the divalent cations Sr^{2+} or Ba^{2+} for extracellular Ca^{2+} *(15,16)*. Ca^{2+} influx is also essential for SV endocytosis in central and peripheral neurons because endocytosis was inhibited by substituting either Ba^{2+} or Sr^{2+} for extracellular Ca^{2+} *(17–19)*, stimulated by adding extracellular Ca^{2+} after SV depletion *(20)*, or stimulated following inhibition of exocytosis by addition of botulinum toxin A *(21)*. The stimulation of SV endocytosis in central nerve terminals requires much lower $[Ca^{2+}]_i$ increases than exocytosis *(19,22)*, indicating that the Ca^{2+} receptor that activates the process has a much higher affinity for Ca^{2+} than the exocytosis Ca^{2+} receptor, thought to be synaptotagmin *(23)*. The higher Ca^{2+} affinity of the endocytosis Ca^{2+} receptor means that SV endocytosis will always be maximally activated by $[Ca^{2+}]_i$ increases that stimulate SV fusion, thus explaining why SV endocytosis was regarded as a Ca^{2+}-independent process.

Is Calmodulin the Stimulatory Ca^{2+} Receptor for Endocytosis?

Endocytosis is inhibited by substitution of either Ba^{2+} and Sr^{2+} for extracellular Ca^{2+} in a number of neuronal preparations, while exocytosis is unaffected. This indicated that the Ca^{2+} receptor that stimulated endocytosis was distinct from the exocytosis Ca^{2+} receptor, synaptotagmin. There is accumulating evidence of a role for the Ca^{2+}-binding protein calmodulin (CaM) in most types of endocytosis in excitable cells since Ba^{2+}, and to a lesser extent Sr^{2+}, block the activation of CaM by Ca^{2+} *(24)*. CaM is essential for rapid/excess retrieval because

pharmacological CaM antagonists, anti-CaM antibodies, and anti-CaM peptides inhibit the process in chromaffin cells *(16).* Further evidence of a role for CaM in endocytosis comes from studies that implicate its downstream targets. These targets are beginning to be identified and initial studies suggest that they differ depending on the type of endocytosis and the type of excitable cell.

Ca^{2+}-Dependent CaM Binding Proteins

CaM controls the function of many proteins in a Ca^{2+}-dependent manner by either: 1) associating with Ca^{2+}-CaM binding proteins or 2) dissociating from CaM-binding proteins on formation of the Ca^{2+}-CaM complex. Some Ca^{2+}-CaM targets seem to have been eliminated from an essential role in most forms of endocytosis, including CaM-dependent kinase II and synapsin I *(16,25),* while other targets such as synaptotagmin and munc-13 remain to be tested *(26,27).* However the association between Ca^{2+}-CaM and two different targets, calcineurin (CaN, the Ca^{2+}-dependent protein phosphatase, PP2B) and the Arp2/3 complex (which is involved in the organization of the cortical actin cytoskeleton) are essential for endocytosis in different systems.

Calcineurin

CaN is a downstream target of the Ca^{2+}-CaM complex *(28).* It is activated by VDCC-mediated Ca^{2+} influx and dephosphorylates a set of nerve terminal proteins called the dephosphins, all of which are essential for SV endocytosis *(29–37).* Thus CaN is the ideal candidate for the final effector in Ca^{2+}-CaM-dependent SV endocytosis. This has been proven in central neurons where the CaN antagonists cyclosporin A and FK506 abolish SV endocytosis, but have no effect on exocytosis in isolated nerve terminals *(19,38).*

How does CaN stimulate SV endocytosis in central neurons? Evidence is now accumulating that CaN exerts its action by dephosphorylating the dephosphins (dynamin I, amphiphysins I and II, synaptojanin, epsin 1, eps15, and AP180). The dephosphins have all been independently shown to be essential for SV endocytosis, and it is becoming clear that their CaN-dependent dephosphorylation also plays an essential role. This is supported by studies that show that an inhibition in the cycle of phosphorylation of two of the dephosphins, dynamin I and synaptojanin, abolish SV endocytosis in central nerve terminals *(38).* Further evidence of an essential role for CaN-mediated dephosphorylation come from studies that show that CaN binds dynamin I in a Ca^{2+}-dependent manner and inhibition of this interaction reduces SV endocytosis in PC12 cells *(39).* The interaction was not phosphorylation-dependent and was maximal at free Ca^{2+} concentrations below 1 μ*M*, which correlates well with the high-affinity Ca^{2+} requirement for endocytosis *(19,22).* CaN may be recruited to the endocytic complex via an interaction between dynamin I and amphiphysin, which may place it in the correct location to dephosphorylate the rest of the dephosphins *(32,40).* This hypothesis is supported by studies that show cain, a protein that binds and inhibits CaN *(41)* also binds amphiphysin I, suggesting that cain may be an endogenous regulator of CaN activity that switches off SV endocytosis *(42).* Overexpression of cain inhibits endocytosis in HEK293 cells, providing further evidence for an inhibitory role *(42).* Cain, dynamin I, and amphiphysin I are also immunoprecipitated together from brain lysates, suggesting the effect of cain was due to an inhibition of CaN activity rather than a disruption of the endocytic complex. How CaN-mediated protein dephosphorylation could stimulate SV endocytosis is still unclear, but may relate to the control of essential protein-protein interactions *(43–45)* or the subcellular localization of the dephosphins *(46,47).*

Various questions remain over the role of CaN in Ca^{2+}-CaM-dependent endocytosis. First, a role for CaM in CaN-dependent SV endocytosis is always assumed but has never been proven. This is now an important question since the discovery of other nerve terminal Ca^{2+}-binding proteins that activate CaN, such

as neuronal Ca^{2+} sensor 1 *(48).* Second, CaN does not seem to have an essential role in endocytosis outside the mammalian CNS. In *Drosophila* NMJ nerve terminals or chromaffin cells, CaN antagonists and anti-CaN antibodies increased, rather than decreased SV endocytosis and rapid/excess endocytosis, respectively *(16,49,50).* The phosphatase also seems to have no essential role in compensatory retrieval, although CaN antagonists do slow its rate in chromaffin cells *(13).* Thus it may be that the essential requirement for CaN is a specialization of SV endocytosis in the mammalian CNS that is not found in other excitable cells.

Actin Cytoskeleton

A Ca^{2+}-CaM interaction with components of the actin cytoskeleton may also be required for endocytosis. Evidence for this originates from studies in yeast where endocytosis is inhibited by the mutation of the single gene that encodes CaM or the gene that encodes Arp2/3, suggesting they both have an essential role *(51,52).* CaM associates with the Arp2/3 complex in Ca^{2+}-dependent manner in vitro and overexpression of CaM in Arp2/3 mutants reverses the inhibition of endocytosis, showing the interaction has a functional role *(52).* CaM is also mislocalised in Arp2/3 mutants, providing further evidence that a Ca^{2+}-CaM association with components of the actin cytoskeleton is essential for endocytosis in yeast.

A role for the actin cytoskeleton in SV endocytosis has also been proposed, because many endocytosis proteins either have roles in actin organization (amphiphysin I, synaptojanin, and syndaptin *(53–55)* or interact with components of the actin cytoskeleton *(55–56).* Also, actin polymerisation was visualized at sites of invaginating membrane using GFP-tagged actin in mast cells *(57)* and F-actin is localized to nerve terminal areas recently proposed to be, "endocytic zones" at frog NMJs *(58).* However, agents that disrupt actin polymerization did not inhibit SV endocytosis either in retinal bipolar neurons or invertebrate NMJ nerve terminals *(59–60).* Thus convincing evidence of an essential role for the actin cytoskeleton in SV endocytosis is still lacking, with or without a requirement for CaM.

Ca^{2+}-Independent CaM Binding Proteins

CaM binds to a group of proteins at resting $[Ca^{2+}]_i$ levels and releases them on formation of the Ca^{2+}-CaM complex. These proteins bind CaM at amino acid regions called "IQ" motifs *(61).* IQ motif proteins, such as VDCCs, GAP-43, and the family of unconventional myosins, are good candidates for effectors of CaM-dependent endocytosis. For example, CaM is proposed to regulate SV endocytosis in hippocampal neurons by releasing GAP-43 on Ca^{2+} influx *(62).* GAP-43 then interacts with rabaptin-5 to stimulate endocytosis. A similar model is proposed in yeast, but with a different effector. As stated previously, yeast CaM mutants cannot perform endocytosis, but CaM mutants lacking a high-affinity Ca^{2+} binding site function normally *(51,63).*

In these mutants an interaction of CaM with unconventional myosin I is essential for endocytosis and this requirement is overcome by deleting the IQ motifs on myosin I *(63).* Thus CaM may have two Ca^{2+}-dependent roles in stimulating endocytosis, a Ca^{2+}-CaM interaction with a target protein, such as CaN or the Arp2/3 complex, and release of an IQ motif protein, possibly an unconventional myosin or GAP-43.

When discussing the requirement for CaM in endocytosis, it should be borne in mind that some studies do not show a requirement for CaM. For example, SV endocytosis was unaffected by CaM antagonists and anti-CaM peptides in retinal bipolar neurons *(6).* However the antagonists were only directed against the Ca^{2+}-CaM complex and not the function of any released IQ motif protein. Additionally a couple of studies in secretory cells have shown that rapid/excess endocytosis can be supported by Ba^{2+} and Sr^{2+} *(64,65).* Thus the role for CaM in most forms of endocytosis in excitable cells is convincing but not yet definitive, and further studies will be required to

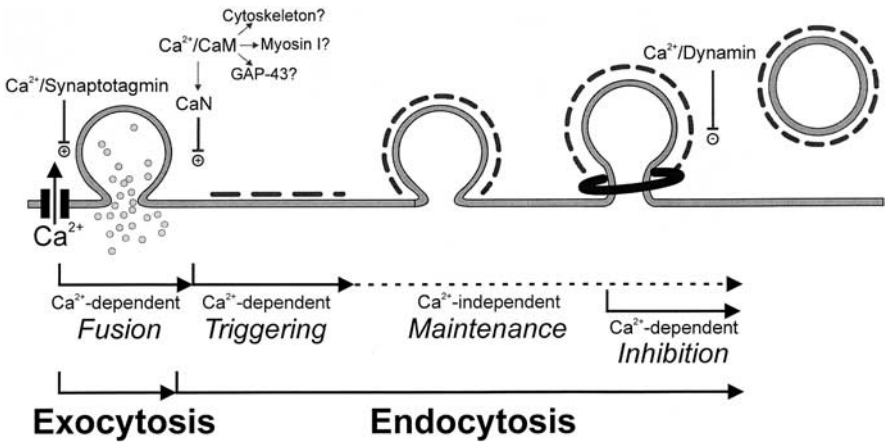

Fig. 1. Ca^{2+}-dependent events during SV fusion and retrieval. On Ca^{2+} influx, Ca^{2+} binds the low-affinity Ca^{2+} receptor synaptotagmin and stimulates SV fusion *(Fusion)* with the plasma membrane. Simultaneously Ca^{2+} binds calmodulin (CaM) to form the Ca^{2+}-CaM complex. This may stimulate SV endocytosis *(Triggering)* in two different ways, by releasing Ca^{2+}-independent binding proteins such as GAP-43 and unconventional myosins, and by activating Ca^{2+}-CaM target proteins such as calcineurin (CaN) and elements of the actin cytoskeleton. CaN then triggers endocytosis by dephosphorylating nerve-terminal proteins possibly via a direct association with dynamin I. After stimulating endocytosis, Ca^{2+} influx is not required for its completion *(Maintenance).* In active zones, where $[Ca^{2+}]_i$ levels increase to μ*M* levels, Ca^{2+} can also inhibit a late phase of endocytosis by blocking the GTPase activity of dynamin I *(Inhibition).*

identify its effectors in these different neuronal systems.

Is Endocytosis Ca^{2+}-Independent?

The evidence presented thus far demonstrates that endocytosis is a Ca^{2+}-dependent process in excitable cells. The question then arises, why did many early studies suggest that SV endocytosis was Ca^{2+}-independent? The answer is twofold. First, SV endocytosis requires much lower $[Ca^{2+}]_i$ increases to be maximally stimulated than exocytosis *(19,22),* giving the appearance of a Ca^{2+}-independent process. Second, most early studies investigated the later stages of SV endocytosis, where the process continues even if Ca^{2+} is subsequently removed. This Ca^{2+}-independent phase is called the maintenance phase (Fig. 1) and was first demonstrated in hippocampal neurons where SV endocytosis was unaffected by the removal of extracellular Ca^{2+} after its stimulation in the presence of Ca^{2+} *(4,5).* The maintenance phase has also been observed in secretory cells where elevation of $[Ca^{2+}]_i$ by caged Ca^{2+} was essential for stimulation of rapid/excess endocytosis, but not for the process to continue *(66)* and in lamprey neurons where extracellular Ca^{2+} was required to trigger, but not to maintain, retrieval of SV membrane from the plasma membrane after intensive stimulation *(20).* Also, studies that arrest SV endocytosis at defined steps found no requirement for Ca^{2+} when the process was restarted. For example, extracellular Ca^{2+} was not required for the completion of SV endocytosis after its arrest at pH- or dynamin-dependent steps in NMJ nerve terminals *(2,49,67).* Finally, modulation of $[Ca^{2+}]_i$ levels during SV endocytosis had no effect its rate at frog NMJ nerve terminals *(68).* All these studies indicate that after the initial stimulation by Ca^{2+} influx, endocytosis proceeds in a Ca^{2+}-independent manner. Thus SV endocytosis is mostly a Ca^{2+}-independent process, but only after stimulation by Ca^{2+} influx.

Does Ca^{2+} Influx Inhibit Endocytosis?

One finding from the early studies remains to be answered, how can VDCC-mediated Ca^{2+} influx inhibit endocytosis when it also stimulates the process? The answer lies in the amount of Ca^{2+} required to inhibit endocytosis. In the first study to demonstrate an inhibition, SV endocytosis was abolished by the elevation of $[Ca^{2+}]_i$ with Ca^{2+} ionophores at ribbon synapses *(6).* However, the $[Ca^{2+}]_i$ increase required for the cessation of SV endocytosis was a higher concentration than most regions of a nerve terminal would ever encounter. For this reason it was assumed that this inhibition would not be physiologically relevant. However nerve-terminal regions called active zones, which have high densities of VDCCs, do encounter high μ*M* levels of $[Ca^{2+}]_i$ on stimulation. These high localized $[Ca^{2+}]_i$ increases (microdomains) are required for exocytosis because synaptotagmin has a very low affinity for Ca^{2+} *(23).* An inhibition of SV endocytosis by μ*M* $[Ca^{2+}]_i$ levels at the active zone has been demonstrated in isolated nerve terminals. In that study the relative amount of SV endocytosis was increased by inhibition of VDCCs at the active zone or by rapid chelation of Ca^{2+} that enters through these VDCCs, thus relieving a tonic inhibition of SV endocytosis by microdomains of high $[Ca^{2+}]_i$ *(22).* This dual inhibition/stimulation of SV endocytosis was quantified at goldfish ribbon-nerve terminals where SV retrieval was stimulated by $[Ca^{2+}]_i$ concentrations above 0.8 μ*M,* but was blocked by $[Ca^{2+}]_i$ levels greater than 20 μ*M (69).* Thus VDCC-mediated Ca^{2+} influx does stimulate *and* inhibit SV endocytosis in the same nerve terminal. However it is important to stress that inhibition will only occur in regions where $[Ca^{2+}]_i$ increases to μ*M* levels, i.e., active zones during stimulation

What is the Inhibitory Ca^{2+} Receptor for Endocytosis?

The Ca^{2+} receptor that inhibits SV endocytosis must have a low affinity for Ca^{2+} since μ*M* concentrations are required to inhibit the process. Strong experimental evidence suggests that this receptor is dynamin I, whose GTPase activity is essential for the final fission event in endocytosis *(29,30).* Ca^{2+} binds both dynamin I (neuron-specific) and the ubiquitously expressed dynamin II with low affinity (μ*M*) and inhibits their GTPase activity *(22,70).* Importantly, identical concentrations of free Ca^{2+} also inhibit dynamin I vesiculation of lipid in vitro, suggesting it would also block dynamin-dependent SV endocytosis *(22).* This was supported by studies showing dynamin II-mediated transferrin uptake was abolished by μ*M* increases in $[Ca^{2+}]_i$ in HeLa cells *(22).*

Although dynamin I is a strong candidate for the inhibitory Ca^{2+} receptor, its role has not yet been proven. Other possible candidates do exist, such as synaptotagmin. Synaptotagmin is essential for SV endocytosis as well as exocytosis, because disruption of the function of its cytosolic C2B domain abolishes endocytosis in a number of systems *(71–73).* The C2B domain is proposed to be a nucleation point of SV endocytosis, because it binds the clathrin adaptor AP-2 *(74,75).* This region also binds phosphatidylinositol (3,4,5) triphosphate [PtdIns(3,4,5)P_3] at resting $[Ca^{2+}]_i$ levels, but in μ*M*-free Ca^{2+}, its specificity of binding switches to phosphatidylinositol (4,5) bisphosphate [PtdIns(4,5)P_2] *(76).* Thus synaptotagmin should compete for nerve-terminal PtdIns(4,5)P_2 when $[Ca^{2+}]_i$ increases to μ*M* levels. This is important because an interaction between dynamin I and PtdIns(4,5)P_2 is essential for SV endocytosis *(77–79).* Thus synaptotagmin may inhibit SV endocytosis at active zones by sequestering PI(4,5)P_2. However the role of synaptotagmin or dynamin I as the inhibitory receptor for SV endocytosis remains to be definitively proven.

Implications for Neuronal Function

How does the regulation of endocytosis by Ca^{2+} shape neuronal function? The consequences and implications of the dual stimulation/inhibition of endocytosis by Ca^{2+} in

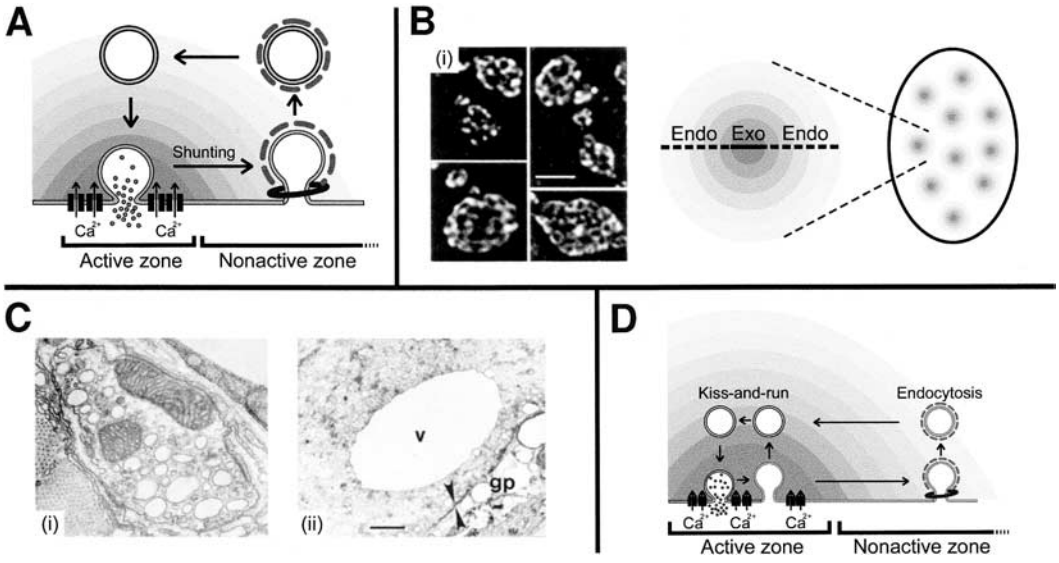

Fig. 2. Ca^{2+} control of endocytosis regulates neuronal function. **(A)** Ca^{2+}-dependent shunting. On Ca^{2+} influx, exocytosis is stimulated and SV endocytosis is arrested by high $[Ca^{2+}]_i$ concentrations at the active zone. SV membrane will be retrieved once it clears the active zone, where the block is relieved and SV endocytosis is stimulated by the lower $[Ca^{2+}]_i$ levels in the nonactive zone. **(B)** Nucleation of endocytosis. (i) In *Drosophila* NMJ nerve terminals the endocytosis protein Dap160 is localized at regions outwith the active zone. (Reprinted from ref. *85;* copyright 1999, with permission from Elsevier Science.) These "endocytic zones" may be located around active zones so they receive the correct concentration of $[Ca^{2+}]_i$ required to stimulate SV endocytosis (high enough to stimulate, but low enough to escape inhibition). This is represented in (ii). **(C)** Repair of membrane damage. Vesiculation induced by injury in squid axons are very similar to cisternae observed after recovery from the permissive temperature in the *shibire Drosophila* mutant, which has GTPase defective dynamin. The massive stimulation of endocytosis seen during axonal injury may in part be due to a relief of a Ca^{2+} inhibition of dynamin GTPase activity. (i) Cisternae produced by a massive stimulation of endocytosis 8 min after return to the permissive temperature in *shibire.* (ii) Vesiculation in squid giant axons 30 min after injury. (Adapted from refs. *90,92,* copyright 1989, 1998, with permission from the Society for Neuroscience.) **(D)** Controlling SV retrieval by multiple mechanisms. High $[Ca^{2+}]_i$ at the active zone may shunt SV endocytosis to nonactive zone areas while simultaneously stimulating kiss-and-run. This model is dependent on SV endocytosis and kiss-and-run having different molecular mechanisms, i.e., Ca^{2+} activating kiss-and-run by accelerating the closure of fusion pores.

neurons is reflected in a model called Ca^{2+} dependent "shunting" (Fig. 2A). Shunting works as follows: activation of Ca^{2+} influx at the active zone will stimulate exocytosis via the low-affinity interaction of Ca^{2+} with synaptotagmin. Simultaneously, SV endocytosis will be arrested via a low-affinity inhibition of dynamin I GTPase by Ca^{2+}. This inhibition will be restricted to the active zone, because microdomains created are transient and $[Ca^{2+}]_i$ concentrations fall exponentially with distance away from these regions. The same VDCC-mediated Ca^{2+} influx will stimulate SV endocytosis in nerve-terminal regions surrounding the active zone where: 1) $[Ca^{2+}]_i$ levels are not high enough to cause inhibition, but 2) are high enough to activate the higher-affinity CaN-dependent pathway *(22).* Thus SV endocytosis is excluded from active zones during stimulation and is "shunted" to surrounding nonactive zone areas by microdomains of high $[Ca^{2+}]_i$ (*22;* Fig. 2A).

Maintenance of Synaptic Transmission

Ca^{2+}-dependent shunting may be a mechanism that regulates synaptic transmission in the short-term by reserving the active zone exclusively for SV exocytosis. The designation of the active zone purely for this function is important in neurons, because the number of sites available for SV docking and fusion is severely limited *(80).* If SV endocytosis were to occur anywhere in the nerve terminal during intense stimulation, synaptic transmission would very quickly cease, because all fusion sites would be occupied by retrieving SVs. Ca^{2+}-dependent shunting should ensure this cannot occur, allowing unimpeded access for incoming SVs that are ready to undergo exocytosis. Thus Ca^{2+}-dependent shunting will maintain the efficiency of synaptic transmission during high-frequency stimulation by allowing a rapid refilling of release sites at the active zone for incoming SVs.

Nucleation of Endocytosis

Receptor-mediated endocytosis is nucleated at defined regions where ligand-bound receptors cluster together to form coated pits *(81).* Evidence is growing that SV endocytosis is also nucleated at defined regions in nerve terminals. Studies in lizard NMJ nerve terminals have shown that SV endocytosis occurs at sites close to the exocytotic active zone *(82).* Also, at NMJ nerve terminals of *Drosophila,* endocytosis proteins are located at defined regions that surround, but do not include, the active zone *(83–85).* The distribution of the identified proteins at these regions does not change on stimulation, suggesting they are permanently anchored to surround exocytotic active zones. It is tempting to speculate that these regions are located in this "doughnut" configuration so that they are in the perfect position to receive the correct amount of free Ca^{2+} (100 nM–1 μ*M*) to stimulate SV endocytosis (Fig. 2B). Thus it seems eminently possible that the location of nucleation points for SV endocytosis, or "endocytic zones," is determined by the concentration of free Ca^{2+} required to trigger the process.

Repair of Neurons

The control of endocytosis by Ca^{2+} may also underlie mechanisms for cellular injury and repair. On plasma-membrane damage, high-localized $[Ca^{2+}]_i$ gradients are created at sites of injury, and large membranous vesicles (cisternae) are formed in a variety of excitable cells, including neurons *(86–89).* These vesicles mostly originate from the plasma membrane by endocytosis and mediate repair by patching the damaged membrane *(90,91).* Ca^{2+} seems to stimulate endocytosis in these damaged regions because injured squid giant axons showed no vesicle production or membrane repair in the absence of extracellular Ca^{2+}, but both processes were reactivated by internal Ca^{2+} perfusion *(90).* Thus Ca^{2+} may mediate membrane repair by blocking endocytosis at the site of injury, while stimulating endocytosis in more distal regions. As these vesicles begin to patch the damaged membrane, $[Ca^{2+}]_i$ levels should decrease, relieving the inhibition of dynamin GTPase activity, and a massive stimulation of endocytosis should occur throughout the injured region to complete the repair. Similar cisternae to that observed in injured axons are also seen in the nerve terminals of the temperature-sensitive *Drosophila* mutant *shibire.* Cisternae appear in *shibire* during a massive stimulation of endocytosis that occurs on return to the permissive temperature after a dynamin-dependent block (*92,93;* Fig. 2C). Considering the morphological similarities between these two different systems it will be interesting to examine whether "collared pits" (intermediates of endocytosis frozen at a dynamin-dependent step in *shibire*) can be seen close to sites of acute nerve injury, which would confirm whether high $[Ca^{2+}]_i$ levels were blocking dynamin GTPase activity.

Regulation of Different Types of Membrane Retrieval in the Same Nerve Terminal

It is becoming apparent that different mechanisms of SV retrieval exist even within the same nerve terminal. In secretory cells a mechanism called "kiss-and-run" occurs where vesicles are retrieved by the closure of the exocytotic fusion pore rather than by conventional clathrin-dependent endocytosis *(94,95).* In chromaffin cells, a transition to kiss-and-run is evoked by stimulating in the presence of high extracellular Ca^{2+} *(96).* Ca^{2+} mediates this transition by accelerating the closure of the exocytotic fusion pore, thus causing the vesicle to detach from the plasma membrane more quickly. Increased extracellular Ca^{2+} also accelerated SV retrieval in cultured hippocampal neurons, suggesting that a similar transition to kiss-and-run may also be stimulated in the mammalian CNS *(97).* Further evidence of a role for Ca^{2+} in a neuronal form of kiss-and-run came from studies at the squid giant synapse. In that study, clathrin-independent SV endocytosis, which may be similar to kiss-and-run, was inhibited when an interaction between the SV protein synaptophysin and dynamin I was disrupted *(98).* Because this interaction only occurs at free Ca^{2+} concentrations above 150 μ*M*, this mechanism of SV retrieval could only take place at the active zone during stimulation.

How can Ca^{2+} stimulate SV retrieval by kiss-and-run while inhibiting SV endocytosis via a block of dynamin I at the active zone? It has been proposed that high $[Ca^{2+}]_i$ levels may hold dynamin I in a GTP-bound "active" state at the active zone. This would allow formation of dynamin I rings that would mediate SV fission when $[Ca^{2+}]_i$ levels decrease *(98).* However this scenario would very quickly block all SV fusion sites, leading to the abolition of synaptic transmission. Also collared pits have never been observed at the active zone during physiological stimulation *(93,99).* A more likely explanation is that kiss-and-run and SV endocytosis operate in tandem in nerve terminals *(93).* This occurs in isolated nerve terminals where changes in the mechanism of stimulation recruit either conventional SV endocytosis or a combination of SV endocytosis and kiss-and-run *(100).* In this model VDCC-mediated Ca^{2+} influx will stimulate kiss-and-run at the active zone while conventional SV endocytosis will be shunted to surrounding nonactive zone regions (Fig. 2D). This model is dependent on these retrieval mechanisms having different molecular components, with high $[Ca^{2+}]_i$ simultaneously closing fusion pores in kiss-and-run while shunting SV endocytosis to nonactive zone areas. Proof of this concept, however, will rely on a demonstration that kiss-and-run is a distinct molecular process and not just a rapid form of SV endocytosis.

Conclusions

It was not very long ago that the major role assigned to Ca^{2+} influx in nerve terminals was to stimulate neurotransmitter release. However, more and more roles for Ca^{2+} influx are becoming apparent in the complexities of the SV life cycle. In addition to the newly identified functions for Ca^{2+} in SV endocytosis and kiss-and-run, Ca^{2+} influx also controls SV trafficking in nerve terminals, by stimulating the refilling of SV pools such as the readily releasable pool and reserve pool *(101–104).* Ca^{2+} has also been proposed to have novel roles in synaptic depression *(105,106)* and in increasing the efficiency of exocytosis (augmentation) *(107).* Add these tasks to the numerous essential and regulatory functions proposed for Ca^{2+}-binding proteins in SV fusion *(108–112)* and it seems that Ca^{2+} influx does indeed work overtime when activated at nerve terminals!

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