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# Multiple Functions of the Vesicular Proton Pump in Nerve Terminals

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**Synaptic vesicles are acidified by a proton pump (vATPase), which allows vesicular uptake of neurotransmitters. After vesicle exocytosis, continued operation of the vATPase would seem to serve no useful function. In this issue of *Neuron*, however, Zhang and colleagues show that continued pumping alkalinizes the cytoplasm, accelerating endocytosis.**

Increasing evidence suggests that the well-known action of the vesicular proton pump (vATPase) in acidifying synaptic vesicles is perhaps not the entire story of its interesting life. In addition to recent suggestions of its effects on SNARE complex formation and fusion pore formation, now comes evidence that its postexocytic pumping of protons out of the cell accelerates endocytosis.

Previous studies have demonstrated an activity-dependent acidification of cytoplasm in cell bodies and dendrites of neurons. The work of Zhang et al. (2010), presented in this issue of *Neuron*, is the first to measure pH changes in mature nerve terminals resulting from nerve activity. Using a transgenic mouse expressing soluble Yellow Fluorescent Protein (YFP, whose fluorescence is quenched by protons) in its motor nerve terminals, they confirm that repetitive stimulation (50 Hz) produces fast acidification like that observed in cell bodies and dendrites. However, they also show that, unlike what happens in other parts of the cell, in the presynaptic terminal this acidification is soon swamped by a much larger alkalinization of the cytoplasm, which decays slowly after stimulation ends. Zhang et al. dissociated this bimodal change in intracellular pH into its two oppositely directed components. The late alkalinization was blocked by poisoning exocytosis with botulinum toxin, and the remaining acidification then followed a simple time course, which resembled the time course of intracellular global calcium ion concentration, rising quickly to a plateau during repetitive stimulation, and falling promptly when stimulation ended. The acidification was completely blocked by preventing calcium entry during stimulation, and the authors propose that it arises mainly from the action of the surface membrane $Ca^{2+}$-ATPase, which, as it pumps calcium ions out of the cell, imports protons. This result is like that observed in neuronal cell bodies and dendrites.

The subsequent alkalinization, however, is an altogether new finding. The fact that it was $Ca^{2+}$ dependent and abolished by botulinum toxins suggested that it arose from the exocytic transfer of the vATPase to the surface membrane, where it continued to pump protons, now against a smaller electrochemical gradient out of the cytoplasm, into the synaptic cleft. Consistent with this, the time course of the alkalinization, in particular its slow decay after tetanic stimulation ended, was similar to the time course of endocytosis (Tabares et al., 2007), suggesting that alkalinization ended as the vATPases were retrieved from the surface membrane by endocytosis.

The continued action of a vesicular membrane protein after its exocytic insertion in the surface membrane, here the vATPase, is reminiscent of studies of "nonquantal release" of the neurotransmitter acetylcholine (ACh), which can be detected (after blocking the extracellular degradation of ACh by the enzyme ACh-esterase) by a small, curare-induced hyperpolarization of the postsynaptic muscle fiber (Katz and Miledi, 1977; Vyskocil et al., 2009). This nonquantal leak of ACh was proposed to reflect the activity of the vesicular ACh transporter when it resides in the surface membrane, presumably after exocytosis. While several possible roles have been proposed, the significance of nonquantal leak of ACh remains unknown. In the retina, on the other hand, evidence shows that nonquantal release ("transport shuttle") of GABA plays an important signaling role (reviewed in Schwartz, 2002).

While the physiological role of ACh transporters during their temporary sojourn in the surface membrane is unclear, the proton pump's activity while there, as shown by the work of Zhang et al., alkalinizes the cytoplasm, which might be significant in regulating endocytosis. For example, blocking activity-induced cytoplasmic alkalinization with folimycin or bafilomycin, two selective inhibitors of the proton pump, reduced the endocytic uptake of FM1-43, suggesting that cytosolic alkalinization promotes endocytosis. The authors point out that this effect could be due to the demonstrated pH sensitivity of internalization of clathrin-coated pits and of dynamin-adaptin binding. Moreover, cytosolic acidification has previously been shown to inhibit endocytosis (Coleman et al., 2008). Thus, the bimodal pH response (acidification followed by alkalinization) observed by Zhang et al. may result in a certain amount of endocytosis inhibition during the first part of prolonged nerve stimulation, followed by endocytosis activation during the rest of the stimulation and for tens of seconds during the post-stimulation period. Zhang et al. also note that presynaptic P/Q-type calcium channels might be inhibited by acidification, and therefore the observed alkalization may prevent this effect and help maintain

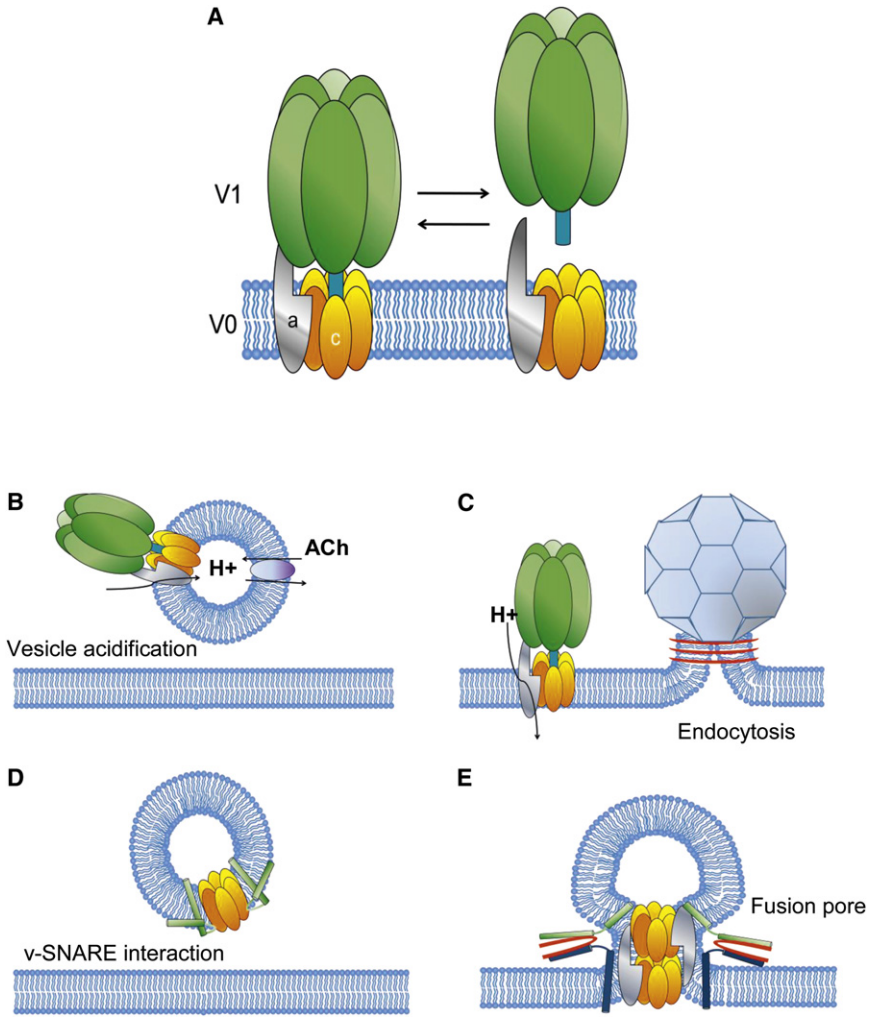


**Figure 1. Proposed Functions of the vATPase in Nerve Terminals**
(A) vATPase is a large enzymatic complex composed of two reversibly associated domains, V0 (in the membrane) and V1 (in the cytoplasm).
(B) Acidification of synaptic vesicles by the vATPase is essential for loading with neurotransmitter.
(C) vATPase continues pumping protons after insertion in the plasma membrane, alkalinizing the cytoplasm, which accelerates endocytosis.
(D) Proposed interaction between the V0 domain and the v-SNARE synaptobrevin.
(E) Suggested role of V0 dimers forming fusion pore in exocytosis, downstream of SNARE complex assembly.

transmitter output during repetitive stimulation.

The changes in cytoplasmic pH were not spatially uniform, which might reflect differences in the density of vATPases in the surface membrane during and after stimulation (differences in the spatial distribution of proton buffers is another possibility). The observed proton "cold spots" are reminiscent of and consistent with the exocytic "hot spots" observed in mice transgenic with synaptopHluorin (Tabares et al., 2007; Gaffield et al., 2009). Such colocalization would be adaptive, in that endocytic rate would be matched favorably to the amount of exocytosis.

Synaptic vesicles in the brain possess one or two copies of the vATPase (Takamori et al., 2006). If the same holds for cholinergic vesicles in motor nerve terminals, then during repetitive stimulation like that used by Zhang et al. (50 Hz for 20 s), which releases about 30,000 quanta, about 45,000 vATPase molecules will be externalized, which with an average presynaptic membrane surface area of 300 $\mu m^2$ would produce a density of 150 proton pumps per $\mu m^2$. (The actual density will be slightly less than this, owing to endocytosis during the 20 s stimulus train; Tabares et al., 2007.) This density is within the range reported for nerve terminals in the electric organ of Torpedo (mean of 40 V0 domains/$\mu m^2$, range up to 200 per $\mu m^2$; Morel et al., 2003).

## The Structure of the vATPase

The vATPase is a multimeric protein complex (Figure 1A) formed by multiple different subunits expressed in all eukaryotic cells. It functions as a proton pumping rotary nanomotor. It is present in intracellular membrane compartments, including synaptic vesicles. The vATPase consists of two multisubunit parts that associate reversibly: V0 is in the membrane and can form a pore, while V1 is in the cytoplasm and is an ATPase (Nishi and Forgac, 2002). Bound and working together, they pump protons into the vesicle. The V0 domain contains a proteolipid oligomer of several *c* subunits and one copy each of subunits *a*, *d*, *e*, and *c″* (Nishi and Forgac, 2002). Different vATPase isoforms exist in mammalian plasma membranes, named by their *a* subunit subtype, whose expression is tissue- and membrane compartment-specific. The *a1* isoform is found in synaptic vesicle membranes (Morel et al., 2003), which is also present in the presynaptic membrane.

## Functions of the Proton Pump

In addition to having a role in acidifying intracellular membrane-bound compartments (Figure 1B), Zhang et al. provide evidence that vATPase speeds up endocytosis by alkalinizing the cytoplasm (Figure 1C). This protein, or several of its subunits, may also have other functions related to exocytosis. The V0 domain of the vATPase interacts with another protein of the synaptic vesicle membrane, synaptobrevin (Figure 1D), one of the core SNARE proteins, and with the SNARE complex in different model systems (Galli et al., 1996; Morel et al., 2003). Recently, Di Giovanni et al. (2010) have demonstrated a $Ca^{2+}$/calmodulin regulated direct protein-protein interaction in synaptic vesicles between synaptobrevin (the v-SNARE) and the *c* subunit of V0.

Furthermore, the perturbation of this interaction produces a substantial decrease in the probability of neurotransmitter release. It has been suggested (Di Giovanni et al., 2010) that the *cis* interaction between synaptobrevins and the *c* subunits of the V0 domain may prevent the formation of the SNARE complex, which implies that dissociation of this complex (regulated by $Ca^{2+}$/calmodulin) must precede fusion. Under this hypothesis it may also be possible that the *c* subunits may help orient synaptobrevin molecules as they enter SNARE complexes with SNAP-25 and syntaxin.

More than two decades ago, Israel et al. (1986) reported the isolation of a proteolipid pore complex (*c* subunit), which they named mediatophore, from synaptosomes formed from Torpedo electroplaques, and they suggested that it mediates calcium-dependent ACh release. Since then, additional evidence has accumulated that the V0 domain of the vATPase participates in membrane fusion downstream of SNAREs (Peters et al., 2001; Hiesinger et al., 2005). One idea is that after a vesicle is fully loaded with neurotransmitter, the cytoplasmic V1 domain dissociates from the intramembrane V0 domain of the vATPase. The naked V0 domain can then dimerize with another V0 domain located in the plasma membrane, and (like a gap junction) create a pore that allows the passage of neurotransmitter from vesicle lumen to synaptic cleft (Figure 1E). Recent reports support this hypothesis. For example, in a loss-of-function mutation in *Drosophila* V0 domain, neurotransmitter loading and synaptic vesicle acidification were not altered, while synaptic vesicle fusion with the presynaptic membrane was blocked downstream of the SNARE complex formation (Hiesinger et al., 2005). It is been proposed that the SNARE complex helps to align the two opposed V0 proteolipid rings, which, when joined together, participate in the formation of the fusion pore.

In summary, in addition to occupying the well-established role of the vesicular proton pump in acidifying synaptic vesicles, accumulating evidence suggests that it, or part of it, participates in other functions related to vesicular trafficking within both the exocytic and endocytic pathways.

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