Chapter 7
The Synaptic Vesicle V-ATPase: A Regulatory Link Between Loading and Fusion?

Oussama El Far and Michael Seagar

Abstract The vacuolar proton pump (V-ATPase) is a huge multi-subunit complex composed of two distinct non-covalently associated sectors. The cytosolic V1 sector hydrolyses ATP, providing the energy for the V0 membrane sector to translocate protons into the vesicle lumen. The proton gradient is then used by vesicular transporters to load synaptic vesicles with specific neurotransmitters. The primary role of the V-ATPase in vesicle loading is widely accepted. However, multiple studies in a variety of model organisms point to an additional general role of the V0 sector in downstream events, notably in regulating SNARE-mediated membrane fusion. This chapter outlines the molecular pharmacology of the V-ATPase and its role in the synaptic vesicle cycle. It then focuses specifically on molecular interactions between V0 subunits and synaptic vesicle trafficking proteins and reviews their relevance to late steps in neurotransmitter release. While this secondary role for the V-ATPase membrane sector is not yet fully established, we speculate that it could provide a regulatory link between vesicle filling and fusion, acting as a filter that allows loaded vesicles to engage the fusion machinery.

Keywords V-ATPase • SNARE proteins • Membrane fusion • Neurotransmitter release • Proton pump

7.1 Introduction

Early observations (Del Castillo and Katz 1954; Heuser et al. 1974; Peper et al. 1974) established the quantal theory, whereby neurotransmitters are released in discrete packets, and proposed synaptic vesicle content to be the functional unit

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of neurotransmission. Nerve endings contain synaptic vesicles, spherical organelles of 30–40 nm in diameter, that fuse with the plasmalemma in response to an increase in intra-terminal calcium concentration, releasing neurotransmitters into the synaptic cleft. Vesicles dock with the presynaptic plasma membrane and acquire precise molecular characteristics that render them responsive to sudden local increase in calcium concentration (Neher and Sakaba 2008), the amplitude of which is determined both by the distance that separates the calcium channel from the releasable vesicle and the calcium-buffering properties of the cytoplasm. Membrane fusion is an extremely rapid process, leading to merging of the synaptic vesicle and plasma membranes and exocytosis of the luminal contents into the synaptic cleft.

An average synaptic vesicle contains 1–2 copies of the vacuolar proton pump (V-ATPase). The V-ATPase consumes ATP to pump protons into the synaptic vesicle lumen. Specific vesicular transporters then use the proton gradient to load vesicles with different neurotransmitters (glutamate, acetylcholine, GABA, glycine, etc.). Following vesicle filling, findings from a variety of model systems suggest that the V0 membrane sector plays an additional role at late steps in exocytosis, notably by interacting with SNAREs. Thus, the V-ATPase potentially provides a regulatory link between loading and exocytosis that is common to all synaptic vesicles, in spite of the diversity of their contents. In this chapter, we will highlight studies to investigate the implication of the membrane sector (V0) of the V-ATPase in SNARE-dependent neurotransmitter release.

7.2 V-ATPase: Molecular Structure and Pharmacology of a Vesicular Proton Pump

V-ATPase is a specialised 900 kDa enzyme complex (Fig. 7.1), containing at least 14 different subunits, some of which are present as multiple copies. Its primary function is to translocate protons and acidify intracellular compartments, using a rotatory mechanism driven by ATP hydrolysis. The V-ATPase complex is made of two distinct sectors V0 and V1. The former is a membrane-embedded 260 kDa oligomer, which constitutes a discontinuous transmembrane pathway for proton transport. It is reversibly associated with a 650 kDa cytosolic V1 sector that carries the ATPase activity (Nishi and Forgac 2002). Electron microscopy studies show V0 has a flexible hexameric doughnut-like structure, (Wilkens et al. 1999; Clare et al. 2006; Wilkens and Forgac 2001), associated with a spherical extramembranous V1 sector (Wilkens et al. 1999). As this chapter will focus on the role of the V0 sector in vesicle trafficking and fusion, we will briefly outline the molecular properties of the V0 subunits.

V-ATPase subunits (Fig. 7.1a) are conventionally designated by ATP6V1 or ATP6V0 followed by letters, using the upper case for V1 (*A–H*) and the lower case

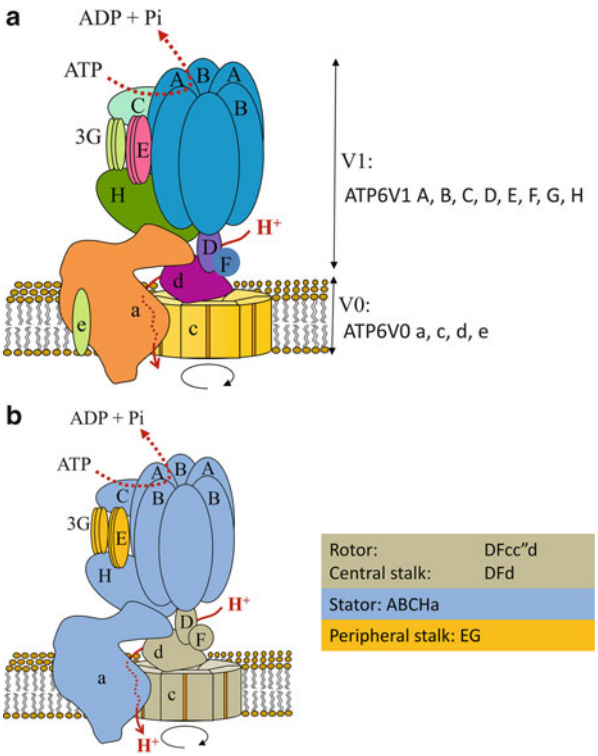

Fig. 7.1 Subunit structure of V-ATPase. (**a**) Schematic representation of V-ATPase subunit structure. The V-ATPase is composed of two reversibly attached sectors, ATP6V1 (extramembranous) and ATP6V0 (transmembranous). (**b**) Schematic representation of V-ATPase functional subdomains. The stator (*blue*) ATP6V1ABCHV0a, the rotor ATP6V1DFV0cc″d and central stalk ATP6V1DFV0d (*grey*) and the peripheral stalk ATP6V1EG (*yellow*). ATP hydrolysis drives rotation of the rotor relative to the stator. The rotation of the c-ring relative to a subunit translocates protons through discontinuous channels from the cytosol into the synaptic vesicle lumen without opening a continuous transmembrane pore

for V0 (a, c, d, e). In higher eukaryotes the V0 sector contains five distinct subunit types a, c, c″, d and e subunits, plus accessory proteins.

V0a subunits (100 kDa) contain a hydrophilic cytosolic N-terminal domain and a C-terminal domain with multiple transmembrane regions (TMR). The membrane topology of the a subunit is still controversial, although recent studies in yeast strongly indicate 8 TMRs. Vertebrate V0a subunits (a1–a4) are coded by four genes. Four a1 splice variants have been identified, and the a1-I form is specifically targeted to nerve terminals (Morel et al. 2003).

V0c (4 TMR, 16 kDa) and c″ subunits (5 TMR, 23 kDa), sometimes called proteolipids, are exceptionally hydrophobic proteins with high sequence similarity that assemble into an oligomeric ring structure containing five copies of c and one copy of c″. Proton transport across the membrane occurs at the interface between transmembrane helices of the a and c subunits. c-subunit helices also constitute the

binding site for the V-ATPase inhibitors bafilomycin and concanamycin (Bowman et al. 2006).

V0d subunit is a soluble 38 kDa protein, which has two isoforms in mammals (d1 and d2), although only the d1 isoform is found in the brain (Nishi et al. 2003). It binds peripherally to the cytoplasmic side of the c-subunit ring and participates in forming the central rotational stalk connecting V1 to the c-subunit ring. This subunit was independently identified as a binding partner for the synaptic vesicle membrane protein synaptophysin and designated physophyllin (Thomas and Betz 1990).

Mammalian V0 may also contain peripheral e1 or e2 subunit isoforms and the accessory proteins Ac45 and M8-9. However, the stoichiometry of these subunits remains to be clarified.

Five principal classes of mammalian V-ATPase inhibitors have been described: plecomacrolide antibiotics, archazolids, benzolactone enamides, indolyls and the so-called late-generation inhibitors (Perez-Sayans et al. 2009). Plecomacrolide antibiotics, mainly bafilomycin and concanamycin, are the most frequently used and best characterised inhibitors. They are active at nanomolar concentrations, bind to the V0c subunit and disrupt proton pumping by inhibiting rotation of transmembrane helices relative to each other in the c-subunit ring.

7.3 The Synaptic Vesicle Cycle: Loading, Release Modes and SNARE Proteins

The classical view of the nerve terminal describes three functionally distinct synaptic vesicle pools (Rizzoli and Betz 2005). (1) The readily releasable pool (≈1 %) corresponds to a few loaded, docked and primed vesicles that await only Ca^{2+} influx to trigger exocytosis of their contents. (2) The recycling pool (10–15 %), under stimulation at physiological frequencies, undergoes continuous cycles of docking, fusion, endocytosis and loading. (3) Finally the reserve pool accounts for 80–90 % of the total population and corresponds to vesicles that only respond to intense stimulation. This pool is probably mobilised when the recycling pool is depleted.

Vesicles in the recycling pool are loaded by concerted action of the V-ATPase and vesicular neurotransmitter transporters (see following Sect. 7.4). Their entry into the readily releasable pool involves SNARE interactions at the vesicle/plasma membrane interface. Briefly, syntaxin 1 and SNAP-25 in the plasma membrane bind to VAMP2 (synaptobrevin) in the vesicle membrane. Trimeric SNARE complexes in a trans configuration constitute a minimal fusion machine, in which helical zippering overcomes lipid repulsion, pulling opposing membranes into close proximity. This is thought to result in hemifusion (Hernandez et al. 2012; Schaub et al. 2006), in which the outer leaflet of the vesicle has merged with the inner leaflet of the plasma membrane. Hence the lumen of a readily releasable

vesicle is thought to be separated from the synaptic cleft by only a single lipid bilayer. However, recent reports indicate that Ca^{2+}-triggered fusion events must start from point contacts that are not hemifused (Diao et al. 2013) and that local bilayer protrusions from t-SNARE vesicles facing v-SNARE vesicles have an increased membrane curvature that might reduce the kinetic barrier to fusion (Bharat et al. 2014). Throughout these events SNARE interactions are chaperoned, stabilised and regulated by a variety of accessory SNARE-binding proteins (see Chap. 4 by Ira Milosevic and Jakob Balslev Sørensen).

Experiments in vitro with pure recombinant proteins in a controlled lipid environment have furthered our understanding of SNARE function. Upon reconstitution into artificial vesicles, trans-assembly of v-SNAREs and t-SNAREs triggers membrane fusion (Di Giovanni et al. 2010b; Karatekin et al. 2010; Weber et al. 1998). Furthermore when SNARE proteins are expressed on the cell surface (Giraudo et al. 2005), they induce intercellular fusion. Similar to synaptic vesicle fusion with the plasma membrane, in vitro SNARE-mediated fusion can be modulated by different SNARE-interacting proteins such as complexin, Munc13 and Munc18 or calcium sensors such as synaptotagmin and calmodulin (Di Giovanni et al. 2010b; Ma et al. 2013; Schaub et al. 2006; Tucker et al. 2004).

Complexins (also called synaphins) are a family of small soluble acidic proteins. Complexin I and II are mainly neuronal and III and IV are retina specific (Reim et al. 2005). They inhibit full SNARE assembly by binding partially assembled trimeric SNARE complexes (Chen et al. 2002; Reim et al. 2001; Tokumaru et al. 2001) and are involved in controlling neurotransmitter release (Huntwork and Littleton 2007; Maximov et al. 2009; Tokumaru et al. 2001; Xue et al. 2007, 2010). The C-terminal domains of complexins I and II have lipid-binding properties (Diao et al. 2013; Malsam et al. 2009; Wragg et al. 2013) and directly bind synaptotagmin 1 in a calcium-enhanced manner (Tokumaru et al. 2008). Although synaptotagmin can directly bind to the assembled SNARE complex, independently from its interactions with complexin (Davis et al. 1999; Tokumaru et al. 2008), complexin recruits Ca^{2+}/synaptotagmin through its C-terminal domain to trigger membrane fusion (Tokumaru et al. 2008). This interaction is thought to release complexin-induced inhibition after Ca^{2+} entry (Schaub et al. 2006; Tang et al. 2006).

In the recent years a complex network of dynamic protein interactions at the active zone, which control different steps in the synaptic vesicle cycle, has been elucidated. These findings, which will be described in detail elsewhere (see Chap. 4 by Ira Milosevic and Jakob Balslev Sørensen), have led to a profound breakthrough in the understanding of synaptic vesicle docking and maturation, as well as the calcium dependency of exocytosis and endocytosis. However, the precise molecular events that result in membrane fusion are less clear.

Synaptic vesicle contents initially escape via a channel-like structure or fusion pore, which perforates the lipid diaphragm separating the vesicle lumen from the surface of the nerve terminal. Using SNARE proteins in opposing lipid membranes, coarse-grained molecular dynamic simulations showed that splayed lipids represent the main barrier in membrane fusion and the trans SNARE complex probably plays a crucial role in the consecutive steps from membrane contact to fusion pore

expansion (Risselada and Grubmüller 2012). Although the implication of SNARE proteins in membrane fusion was part of the discoveries rewarded by the 2013 Nobel Prize in Physiology or Medicine, the detailed molecular processes that take place during the final step in synaptic vesicle/plasma membrane fusion remain enigmatic. Functionally distinct SNARE-mediated release modes have been described, although the molecular mechanisms that underlie transitions between these different release modes are unknown. A "full fusion" mode leading to a complete collapse of the synaptic vesicle membrane into the nerve terminal plasma membrane contrasts with the so-called kiss and run fusion mode in which synaptic vesicles partially release their contents through a fusion pore that can open and close. In order to account for the reversal of the fusion pore opening, it has been suggested that a protein-based scaffold might inhibit its expansion or even constitute a fusion pore gating mechanism (Almers and Tse 1990; Chernomordik and Kozlov 2008). In 2001, Peters et al. suggested that the V0 sector of the V-ATPase could play this kind of role in yeast vacuole fusion. As basic membrane fusion events are conserved from yeast to man and ruled by similar molecular mechanisms (Ferro-Novick and Jahn 1994), this idea has been extrapolated to synaptic vesicle fusion (Morel et al. 2001). However, the conceptual leap from yeast to the nerve terminal must also account for numerous specialisations, including transitions between different release modes, spontaneous and asynchronous release as well as the extreme rapidity of synchronous synaptic release.

7.4 V-ATPase and Vesicular Transporters in Vesicle Loading

The eukaryotic V-ATPase is located on intracellular membrane compartments, including synaptic vesicles, where it couples ATP hydrolysis to proton pumping into the lumen. However, the V-ATPase can also be expressed either constitutively (Breton et al. 1996; Schlesinger et al. 1997; Wagner et al. 2004) or in a transient manner (Zhang et al. 2010) at the plasma membrane of certain specialised cells where it contributes to extracellular acidification (Breton et al. 1996; Schlesinger et al. 1997; Wagner et al. 2004) and intracellular alkalinisation (Zhang et al. 2010).

As well as the two reversibly associated V1 and V0 sectors, the V-ATPase can be divided into functional subdomains (Fig. 7.1b). A V1DFV0cc″d complex constitutes the rotor: comprised of the c-ring (V0cc″) and the central stalk (V1DFV0d). The rotor is surrounded by the stator: comprised of the catalytic V1AB hexamer and the collar (V1CHV0a). The catalytic hexamer and the collar are connected by peripheral stalk V1EG complexes. Hydrolysis of ATP by the V1AB hexamer drives rotation of the rotor relative to stator. Rotation of the V0c-ring relative to V0a subunit translocates protons at their interface, from the cytosol into the synaptic vesicle lumen. Exactly how protons transit the membrane is not known. However, the dominant hypothesis postulates two offset

hemi-channels. The first hemi-channel delivers protons from the cytosol to a conserved glutamate residue located in c-subunit TMR 4 at the periphery of the c-ring, the second from the c-ring to the lumen. Thus transport is achieved without opening a continuous transmembrane pore.

Proton influx acidifies intracellular compartments (pH 5.2–5.5). Furthermore the proton gradient also actively participates in generating synaptic vesicle membrane potential ($\Delta\Psi$). Specific neurotransmitter transporters in the vesicle membrane use either $\Delta\Psi$ for glutamate loading or proton cotransport for GABA, acetylcholine and monoamine uptake (Liu and Edwards 1997; Maycox et al. 1990; Moriyama et al. 1992; Nelson and Lill 1994). Acidification by V-ATPase is an important factor in vesicular trafficking and the dynamics of cellular compartments. It regulates membrane protein sorting (Nishi and Forgac 2002) (Marshansky and Futai 2008) and endo-membranous degradation and is involved in autophagy (Williamson and Hiesinger 2010; Williamson et al. 2010).

Reversible dissociation of V1 and V0 sectors arrests proton transport and is a physiological process used to regulate ATPase activity and luminal acidification (Kane 1995; Qi et al. 2007; Sumner et al. 1995). The dissociation of sectors is very sensitive to cellular environment and occurs principally in response to nutrient depletion, probably via a pH-dependent mechanism (Qi et al. 2007). The subunit composition of the V1 sector, namely, V1A isoforms and V1C (Perez-Sayans et al. 2012), determines coupling efficiency and therefore regulates proton transport. The factors that regulate assembly/disassembly have been mainly studied in the lower eukaryotes and in yeast, the a subunit (Vph1 and Stv1) plays a crucial role. Disassembly involves microtubules while assembly relies on the cytosolic Rabconnectins or RAVE (Regulator of H^+-ATPase of Vacuolar and Endosomal membranes) complex. The glycolytic enzyme aldolase, possibly acting as a glucose sensor, also regulates V-ATPase activity by direct interaction with the b subunit and disruption of binding results in disassembly of V1/V0 (Lu et al. 2001, 2007). Furthermore V-ATPase and aldolase can also form a ternary complex with ARNO/Arf6 (Merkulova et al. 2011), proteins which have been implicated in linking intravesicular pH with the regulation of trafficking in the endocytic pathway (Hurtado-Lorenzo et al. 2006). Specifically the recruitment of the small GTPase Arf6 and ARNO (cytohesin-2) from the cytosol to the endosome membrane surface is dependent on acidification of the lumen by V-ATPase. Arf6 and ARNO bind to V0c and V0a subunits, respectively, and disruption of binding inhibited endocytosis, suggesting that intraluminal pH sensing by the V-ATPase can impact membrane trafficking. The relevance of these different pathways (Fig. 7.2) to the regulation of V-ATPase activity and membrane trafficking in the nerve terminal has not yet been explored in detail. However, studies in zebrafish hair cells have shown that rabconnectin3α modulates V-ATPase activity in synaptic vesicles by promoting assembly of V1 and V0 (Einhorn et al. 2012).

Vesicle loading with transmitter thus depends on the V-ATPase, which is a universal component of all synaptic vesicles, acting upstream of the transporter (glutamate, GABA, etc.) that defines specific vesicle content. Thus the V-ATPase is ideally situated to provide quality control of vesicle filling by monitoring and regulating the proton gradient. Presumably it is more cost-effective for the nerve

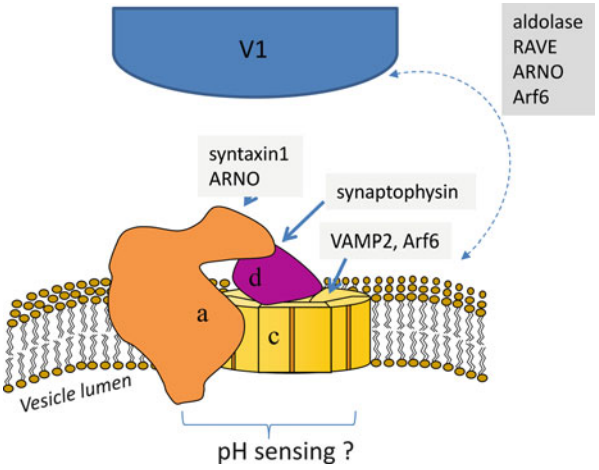

Fig. 7.2 Interactions of metabolic and trafficking proteins with V0 subunits. Schematic representation of molecular interactions involving the V-ATPase V0 sector. Interactions (*arrows*) are represented in two functional categories: those that are implicated in vesicle trafficking and exocytosis (VAMP2, syntaxin1, synaptophysin, ARNO and Arf6) and others involved in control of V1/V0 interactions and pH sensing (ARNO, Arf6, RAVE and aldolase)

terminal to avoid the exocytosis of empty or partially filled vesicles. But how could the trafficking and fusion machinery know when a synaptic vesicle is full? It has been suggested (Morel et al. 2003) that when a vesicle is fully loaded, proton efflux from the lumen stops, because it is no longer required to compensate neurotransmitter influx. Hence maximal acidification is attained, which would result in dissociation of V1. In this way vesicles could sense luminal pH and translate it into a "full" signal at the vesicle surface. Thus isolated V0 sectors, unmasked by V1 assembly, would become available to engage the docking and fusion machinery (Morel et al. 2003) (Fig. 7.3). While this attractive concept seems compatible with the quantal nature of transmitter release, it leads to further challenging questions. Can empty vesicles fuse? When proton transport is blocked, does exocytosis still occur?

Vesicular neurotransmitter concentration itself does not seem to be a limiting factor for fusion (Edwards 2007), as vesicles with a reduced neurotransmitter content still fuse (Zhou et al. 2000). Inhibition of proton transport by bafilomycin A1 leads to diminished activity when postsynaptic responses are used to monitor presynaptic exocytosis (Di Giovanni et al. 2010a; Zhou et al. 2000). However, this does not rule out the possibility that empty vesicles still fuse. More recent studies in the calyx of Held (Xue et al. 2013) using FM dye to monitor vesicle exocytosis showed that in the presence of bafilomycin A1, vesicles fuse and fully participate in the recycling synaptic vesicle pool. These results suggest that unacidified neurotransmitter-free synaptic vesicles can still accomplish fusion. In apparent contradiction with this finding, data from Poëa-Guyon and collaborators support the view that luminal acidification is a prerequisite for fusion (Poea-Guyon et al. 2013). They argue that the V-ATPase has a role in intraluminal pH sensing like it does in endosomes (Hurtado-Lorenzo et al. 2006). However, bafilomycin,

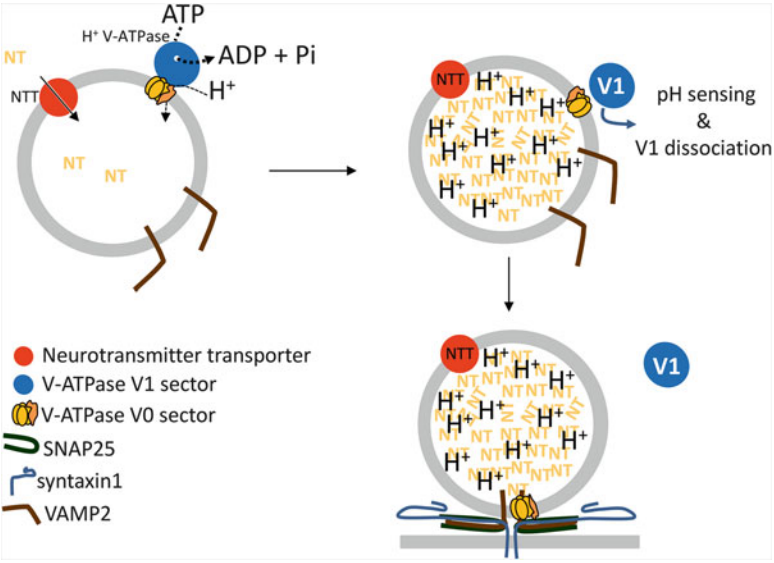

Fig. 7.3 Regulatory links between loading and fusion. ATP-driven H^+ transport through fully assembled V-ATPase at synaptic vesicle membranes is crucial for synaptic vesicle loading with neurotransmitters (NTT). Completion of synaptic vesicle loading leads to dissociation of the V1 sector and renders V0 accessible to bind SNARE proteins. V0 sector is speculatively located near the fusion pore

which binds to V-ATPase c-subunit, promotes V1 disassembly and stabilises a conformation of V0 that mimics a maximally acidified "ready for exocytosis" state.

7.5 A Role for V0 Subunits in Membrane Trafficking and Exocytosis

In the preceding section we reviewed evidence that V0 subunits may carry molecular signals (domains unmasked by dissociation of V1, bound ARNO/Arf6, conformational changes intrinsic to V0 subunits) that report intraluminal pH and thus indirectly monitor loading. In this section we address the possibility that V0 subunits play a direct role in docking and fusion downstream of loading. This role can be viewed from different angles: pore-formers and/or lipid destabilisers and SNARE partners.

7.5.1 Pore-Formers and/or Lipid Destabilisers

From the early 1980s, reports appeared in the literature initially suggesting that the V0 sector of the V-ATPase has a direct role in acetylcholine release. In an attempt at identifying a putative presynaptic translocator of acetylcholine (ACh) that could directly release ACh molecules from the cytoplasm into the synaptic cleft, Israel and collaborators purified a 150–200 kDa protein complex from *Torpedo marmorata* electric organ (Birman et al. 1986) (Israel et al. 1986). This complex was found to be composed of a single very hydrophobic 17 kDa polypeptide, which upon reconstitution in proteoliposomes showed calcium-dependent pore properties. The 17 kDa protein, initially designated "mediatophore", finally turned out to be the V0c subunit of the V-ATPase (Birman et al. 1990). These findings were not pursued by other laboratories since the authors advocated a controversial non-vesicular release model, incompatible with the mainstream view of synaptic vesicle exocytosis.

Although V0c is intrinsically expressed in release-incompetent neuroblastoma cells, overexpression of V0c conferred vesicle-mediated calcium-induced release properties (Bugnard et al. 1999). Most interestingly, freeze-fracture images from these cells were similar to synaptic vesicle fusion events observed much earlier at frog neuromuscular junction (Heuser et al. 1979) or Torpedo synaptic terminals upon neurotransmitter release (Muller et al. 1987). Immunolabelling of freeze-fractured Torpedo electric organ synaptosomes showed the presence of this protein on synaptic vesicles at the docking interface with the plasma membrane (Morel et al. 2001).

Overexpression of V0c in substantia nigra also increased dopamine release. Although the precise molecular mechanism underlying DA release is not yet clear, it has been suggested that overexpressing V0c might be a useful rescue strategy, in addition to enzymes of the dopamine synthetic pathway, for the gene therapy of Parkinson's disease (Jin et al. 2012).

In an independent investigation into how Ca^{2+}/calmodulin (Ca^{2+}/CaM) signals completion of docking and triggering in yeast vacuolar fusion (Peters and Mayer 1998), Peters et al. (2001) reported that the V0 sector is implicated in vacuolar fusion, independently of its proton transport activity, and displays pore-like properties (Almers 2001; Morel et al. 2001; Zimmerberg 2001). They showed that V0c binds Ca^{2+}/CaM and that CaM-dependent V0-V0 trans-complexes assembled after vacuolar docking and downstream of SNARE complex pairing. The presence of yeast V0a subunit, on both vacuoles destined to fuse, was later shown to be required for over 80 % of fusion events (Bayer et al. 2003) and that this protein is involved in calcium efflux from vacuoles, without being part of the Ca^{2+} release channel (Bayer et al. 2003). In photoreceptor synapses, V0a was shown to directly interact with Ca^{2+}/CaM. While binding is not necessary for synaptic targeting of V0a, this investigation uncovered functional regulation of V-ATPase by CaM (Zhang et al. 2008). Furthermore impairment of CaM binding to V0a resulted in dose-dependent neuronal toxicity. It is interesting also to note that V0c was also reported

to be part of connexon-free gap junctions in crustaceans (Dermietzel et al. 1989; Finbow and Pitts 1993). V0a has a regulatory role in insulin secretion as deficiency in the V0a subunit in insulin-containing granules (V0a3) drastically impaired secretion from beta cells in mouse pancreatic islets (Sun-Wada et al. 2006).

Exosomes are small vesicular structures (30–100 nm) contained in endosome-derived multivesicular bodies (MVB) (van Niel et al. 2006). In a variety of cell types including neurons (Faure et al. 2006), MVBs fuse with the plasma membrane and release exosomes into the extracellular milieu. A study on cuticle formation in *C. elegans* uncovered the involvement of vha5 (V0a of *C. elegans*) in MVB fusion with the apical membrane during release of exosomes and hedgehog-related cuticle components (Liegeois et al. 2006). Furthermore microglial phagosome/lysosome fusion in zebrafish was shown to be mediated by V0a, independently of its proton transport activity (Peri and Nusslein-Volhard 2008). In a different register, bone-resorbing osteoclasts are multinucleated, giant cells of haematopoietic origin, formed by the fusion of mononuclear pre-osteoclasts derived from myeloid cells. Deficiency in V0d2 subunit dramatically inhibited pre-osteoclast fusion and therefore bone resorption, independently of V-ATPase proton pump activity (Lee et al. 2006). This study suggests that V-ATPase V0 components are not only involved in intracellular fusion mechanisms but can also be required for cellular fusion.

7.5.2 SNARE Partners

In a search for synaptic vesicle-binding proteins, early studies by Thomas and Betz suggested an interaction of the soluble V-ATPase V0d subunit with synaptophysin, a synaptic vesicle signature protein of unknown function that interacts with VAMP2 (Thomas and Betz 1990). The association of V-ATPase components with SNARE proteins was first reported using immunoprecipitation from rat brain extracts (Galli et al. 1996). In this study using anti-VAMP2 antibodies, several V0 subunits and synaptophysin were co-immunoprecipitated. In elegant differential co-immunoprecipitation studies, a complex of V0a and V0c was found to associate with assembled SNARE complexes, as well as with VAMP2 (Morel et al. 2003). In an unbiased screen for genes implicated in synaptic function in *Drosophila* photoreceptors, V0a subunit was found to be involved in a late step of synaptic vesicle exocytosis and its deficit induces severe defects in evoked synaptic transmission. Interestingly, these effects were independent of V0a action in acidification (Hiesinger et al. 2005). Further investigation of the molecular mechanism underlying these defects demonstrated a direct interaction of the N-terminal part of the t-SNARE syntaxin with V0a. In an attempt to dissect SNARE protein interactions with V0c, the most hydrophobic component of the V-ATPase V0 sector, a direct interaction with the v-SNARE VAMP2 was uncovered (Di Giovanni et al. 2010a). Interaction details were mapped on both binding partners and shown to involve the V0c cytosolic loop that links TMR 3 and TMR 4 and the juxtamembrane VAMP2 tryptophan residues 89 and 90, formerly shown by the same group to mediate

binding to Ca^{2+}/CaM (Di Giovanni et al. 2010a, b). Furthermore, VAMP2 interactions with Ca^{2+}/CaM and V0c are mutually exclusive. Mutating these tryptophan residues inhibited Ca^{2+}-dependent exocytosis in PC12 cells (Quetglas et al. 2002) as well as in neurons (Maximov et al. 2009). Most interestingly, perturbing V0c/VAMP2 binding, using interfering peptides from V0c loop 3.4 or VAMP2 juxtamembrane domain, inhibited glutamatergic as well as cholinergic neurotransmitter release from neurons in cortical slices and cultured SCG. Inhibition was not observed if both peptides were co-injected (unpublished data) or upon mutation of the tryptophan residues in VAMP2 peptide or scrambling the V0c loop 3.4 peptide sequence (Di Giovanni et al. 2010a, b). The 1 VAMP2: 1 V0c binding stoichiometry suggests that the c-subunit ring could organise v-SNAREs into a radial array and determine the number of SNARE complexes that assemble around the fusion pore (El Far and Seagar 2011). In this case, due to intrinsic hydrophilic properties of V0d, the V0a N-terminal domain as well as the extravesicular loops of V0c, hemifusion intermediates are unlikely in fusion events involving V0.

7.6 Conclusions and Future Directions

In this chapter we have highlighted over 30 years of research that converges to support a role for the V0 sector of the V-ATPase in diverse forms of SNARE-mediated fusion: organelle fusion (yeast vacuoles, phagosomes/lysosomes), regulated secretion, exosomal release from MVBs and synaptic neurotransmitter release. These findings are consistent with a model in which V0 function goes beyond that of an ATP-dependent proton pump, to include monitoring of vesicle pH, loading with neurotransmitters and membrane fusion. Implicit in these observations is the hypothesis that intraluminal pH promotes conformational transitions of V0 subunits, which modulate direct interactions with SNAREs and impact the fusion process. If how and when V0 subunits act in membrane fusion are still open to debate. At the interface of membranes destined to fuse, the presence of a structure that geometrically organises a fixed number of trans SNARE complexes could be a simple way to standardise the energy resources available to drive membrane fusion. Alternatively, partial SNARE complex assembly could pin membranes together and provide precisely located scaffolding for proteins that act downstream to form a channel-like pore or catalyse rearrangements of lipid pore intermediates. Models of this kind are not yet energetically delimited, but certainly deserve theoretical investigation. Progress will require answers to some of the following questions. Do V0 subunits provide signals indicating that SNARE assembly can begin? Can they organise SNAREs into radial oligomeric arrays with appropriate properties to overcome energy barriers to membrane fusion? Might this also involve V0 subunits destabilising bilayers to promote opening of a lipid-lined fusion pore? Can pure recombinant V0 subunits form a protein-lined pore? Future investigations will hopefully address some of these issues.

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