Zuerst ersch. in : International Review of Cell and Molecular Biology ; 306 (2013). - pp. 371-416
http://dx.doi.org/10.1016/B978-0-12-407694-5.00009-2

Contractile Vacuole Complex—Its Expanding Protein Inventory

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Contents

Abstract

The contractile vacuole complex (CVC) of some protists serves for the osmotic equilibration of water and ions, notably Ca^{2+}, by chemiosmotic exploitation of a H^+ gradient generated by the organelle-resident V-type H^+-ATPase. Ca^{2+} is mostly extruded, but there is also some reflux into the cytosol via Ca^{2+}-release channels. Most data available

are from *Dictyostelium* and *Paramecium*. In *Paramecium*, the major parts of CVC contain several v-/R-SNARE (synaptobrevins) and t-/Q-SNARE (syntaxins) proteins. This is complemented by Rab-type GTPases (shown in *Tetrahymena*) and exocyst components (*Chlamydomonas*). All this reflects a multitude of membrane interactions and fusion processes. Ca^{2+}/H^{+} and other exchangers are to be postulated, as are aquaporins and mechanosensitive Ca^{2+} channels. From the complexity of the organelle, many more proteins may be expected. For instance, the pore is endowed with its own set of proteins. We may now envisage the regulation of membrane dynamics (reversible tubulation) and the epigenetic control of organelle shape, size and positioning. New aspects about organelle function and biogenesis are sketched in Section 7. The manifold regulators currently known from CVC suggest the cooperation of widely different mechanisms to maintain its dynamic function and to drive its biogenesis.

ABBREVIATIONS

CRC Ca^{2+} release channel
CVC contractile vacuole complex
SNARE soluble NSF [*N* ethylmaleimide sensitive factor] attachment protein receptor

1. INTRODUCTION

Freshwater protists constantly have to cope with the permeation of water into the cell. They have developed a complex organelle, the contractile vacuole complex (CVC), for the extrusion of water and an excess of ions, notably Ca^{2+}. There are general principles in the design of CVC, though with considerable variation (Allen, 2000; De Chastellier et al., 1978; Frankel, 2000; Hausmann and Patterson, 1984; Patterson, 1980; Schneider, 1960). One of the most important feature is the endowment with a V type H^{+} ATPase as a primary active transporter (Fok et al., 1993; Heuser et al., 1993; Nolta et al., 1993). It forms a proton gradient (ΔH^{+}) which chemiosmotically drives the transfer of water and ions into the contractile vacuole (CV). The CV expels its contents by exocytosis at preformed sites, the "pore" (Allen, 2000; Allen and Naitoh, 2002; Frankel, 2000; McKanna, 1973, 1976).

2. BASIC STRUCTURAL AND FUNCTIONAL ELEMENTS OF CVC

The CVCs of different organisms share some important structural and molecular elements. Although some CVC proteins are known only from

one cell type, in most cases, this is evidently due to stochastic data collection, rather than to systematic differences.

2.1. Basic structure of CVC

Although the CVC is basically constructed according to the same principles in different phyla, one can also recognize some differences. In its typical form, for example, in ciliates, CV is connected via ampullae to several radial arms (collecting canals) during diastole; they are transiently disconnected during systole (Tominaga and Allen, 1998). Each of the radial arms is in open connection with a branched tubular network (spongiome) whose proximal part appears smooth in the electron microscope (EM). Branching is best visible in the EM image collection by R. D. Allen, accessible at http://www5.pbrc.hawaii.edu/allen/. This smooth spongiome is connected to the decorated spongiome whose tubules are studded with pegs, first discussed as potential H^+ ATPase molecules (McKanna, 1976). Subsequently, they have been identified as the catalytic V1 head parts of the H^+ ATPase in *Dictyostelium* (Fok et al., 1993; Heuser et al., 1993; Nolta et al., 1993) and in *Paramecium* (Fok et al., 1995; Wassmer et al., 2005, 2006). The smooth spongiome expands upon systole to accommodate the excess of membrane area from collapsing radial arms (Section 4.1). Thus, the smooth spongiome is a flexible membrane reservoir allowing for swelling during diastole in ciliates (Allen, 2000; Frankel, 2000) and in *Dictyostelium* (Gerisch et al., 2002).

In *Dictyostelium* (De Chastellier et al., 1978; Heuser et al., 1993; Zanchi et al., 2010) and in the flagellate green algae *Chlamydomonas* (Luykx et al., 1997), smaller vacuoles are seen to emerge, to swell, and finally to fuse to a large CV whose contents are discharged at the cell membrane. These vacuoles are also surrounded by a spongiome, an anastomosing tubular network. The rather distinct structure of the CVC in *Paramecium* observed at the light (Fig. 9.1) and EM level (Fig. 9.2) can be largely attributed to its support by regularly arranged microtubules extending from the pore out to the tips of radial arms (Schneider, 1960). No such lining is reported from *Dictyostelium*, neither from ultrastructural analysis (De Chastellier et al., 1978) nor from antibody labeling (Gräf, 2009) or expression of GFP (green fluorescent protein) tagged tubulin (Samereier et al., 2011). Nevertheless, there may be some association of the *Dictyostelium* CVC with microtubules because of the disorganizing effect of nocodazole treatment (Jung et al., 2009).

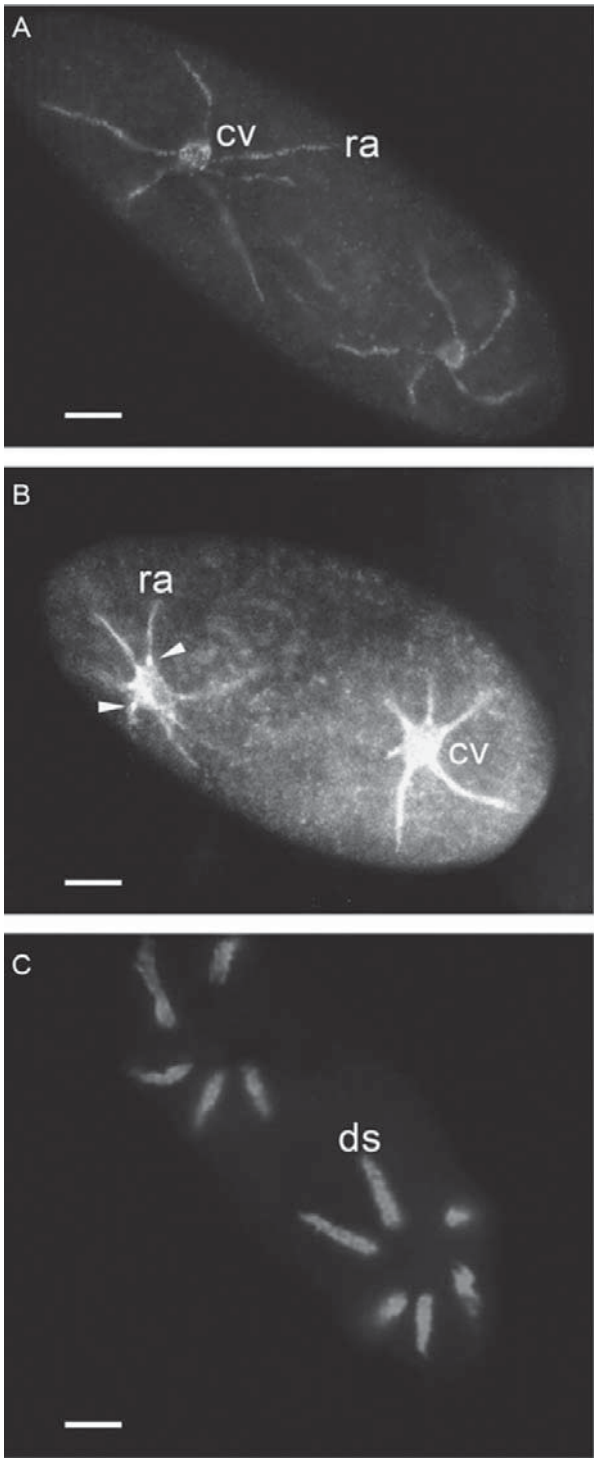

Figure 9.1 Examples of several CVC components of *P. tetraurelia* visualized by fluorescence microscopy. (A) Syb2-GFP stains the CV and radial arms with adjacent smooth spongiome (confirmed by high-resolution immuno-EM). (B) NSF localized by anti-NSF antibodies under precautions allowing for the retention of the antigen at the sites of NSF activity. Note staining of CV and radial arms with adjacent smooth spongiome (evident from comparison with (A)) and hot spots at the CV/radial arms junctions (arrowheads). (C) H^+-ATPase visualized by the F-subunit (of the catalytic V1 part) as a GFP-fusion protein reveals its localization to the decorated spongiome (confirmed by immuno-EM). cv, contractile vacuole; ds, decorated spongiome; ra, radial arm (with decorated spongiome attached). Bars = 10 μm. *(A) From Schilde et al. (2006), (B) from Kissmehl et al. (2002), and (C) from Wassmer et al. (2005).*

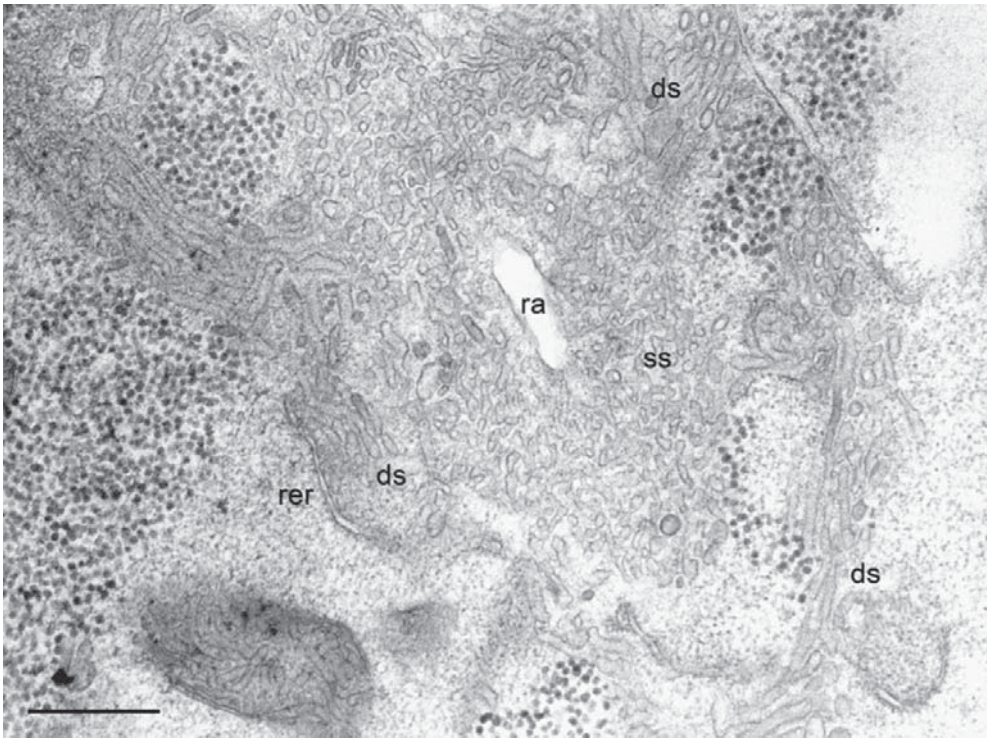

Figure 9.2 Normal EM appearance of the *P. tetraurelia* CVC. Centered around a radial arm (ra) is the labyrinth of the smooth spongiome (ss) which is flanked by tubules of the decorated spongiome (ds) and occasionally by a cisterna or the rough ER (rer) with only few ribosomes attached. Unpublished micrograph. Bar = 0.1 μm.

2.2. Proton pump as a basic constituent

The V type H^+ ATPase consists of two multimeric protein complexes, the transmembrane V0 base piece and the catalytic V1 headpiece which can be reversibly detached and reattached (Hinton et al., 2009). The H^+ ATPase is electrogenic and thus of central importance for CVC function. In *Paramecium* (Grønlien et al., 2002), it has been shown to energize CVC membranes for the—still hypothetic—secondary active transport of water and ions. First discovered in *Dictyostelium* (Fok et al., 1993; Heuser et al., 1993; Nolta et al., 1993), its subunits have been identified successively in *Paramecium* (Fok et al., 1995; Wassmer et al., 2005, 2006). Here, the number of 17 a subunits (SU) is excessive when compared with the four isoforms occurring in the mouse (Wassmer et al., 2005). This SU spans the holoenzyme and thus forms a con necting piece (stalk) between the V0 and the V1 part (Wassmer et al., 2005), as it does in all cells (Lafourcade et al., 2008). Its polymorphy may allow for the selective association of variable other SUs. Also, in *Paramecium,* one par ticular SU isoform, a2 1, is restricted to the CVC (Wassmer et al., 2009). This selectivity may reflect either a selective membrane delivery process in which other CVC components may participate and/or requirement

for pumping kinetics, but neither aspect has been ascertained. The mergence of multiple isoforms of many proteins in *Paramecium* is explained by several rounds of whole genome duplications (Aury et al., 2006).

2.3. Proteins required for membrane trafficking

Tables 9.1 and 9.2 summarize the protein inventory of the CVC in different systems. Evidently current knowledge about molecular components of the CVC is a patchwork, with information about widely different aspects from the different cell types analyzed. Their comparison may promote future analyses.

2.3.1 SNARE proteins

Alone, the reversible fusion/fission processes at the pore and at the CV/radial arms connection call for SNARE proteins (soluble NSF (*N* ethylmaleimide sensitive factor) attachment protein receptors) and the SNARE specific chaperone, NSF. Surprisingly, only cursory information exists apart from *Paramecium*. SNARE proteins are generally known to mediate docking of a vesicle to a target membrane by formation of a trans complex and finally fusion (Jahn and Fasshauer, 2012; Jahn and Scheller, 2006). Depending on whether located at the vesicle or at the target side, one differentiates between v /R SNAREs and t /Q SNAREs; R and Q indicate the central amino acid in the α helical SNARE domain. In *Paramecium*, several t /Q SNAREs and v /R SNAREs as well as NSF are localized to the CVC. These CVC resident SNAREs in *P. tetraurelia* (*Pt*SNAREs) encompass the t SNAREs Syntaxin2 (Syx), Syx14, and Syx15 (Kissmehl et al., 2007; Schönemann et al., 2013) and the broadly distributed t /Qab SNARE type SNAP25 like protein (LP) (Schilde et al., 2008) as well as the v /R SNAREs Synaptobrevin2 (Syb), Syb6, and Syb9 (Schilde et al., 2006, 2010; Schönemann et al., 2013). In *Paramecium*, synaptobrevins are actually longins, just as in plants (Plattner, 2010a,b).

Whole cell surface capacitance measurements have documented the reversible detachment/reattachment of radial arms (with their ampullae) during each pumping cycle of the CVC in *Paramecium multimicronucleatum* (Grønlien et al., 2002; Tominaga and Allen, 1998). Here, NSF has been localized in gently saponin permeabilized, surviving *P. tetraurelia* cells by adding antibodies against species specific NSF in presence of the inhibitor, *N* ethylmaleimide, and nonhydrolyzable ATP γ S (Kissmehl et al., 2002).

The occurrence of NSF and SNAREs over large parts of the CVC, particularly in the smooth spongiome, suggests the occurrence of many

Table 9.1 Components of contractile vacuole complex localized to the CVC of *Paramecium*[a]

Components	Method	Reference
SNARE-specific chaperone		
NSF	Immuno LM	Kissmehl et al. (2002)
SNAREs		
Syb2	GFP, immuno LM	Schilde et al. (2006)
	GFP/immuno EM	
Syb6	Immuno LM/ EM	Schönemann et al. (2013)
Syb9	Immuno LM/ EM	Schönemann et al. (2013)
Syb10	Immuno LM	Schilde et al. (2010)[b]
Syx2	GFP	Kissmehl et al. (2007)
Syx14	GFP	Kissmehl et al. (2007)
	Immuno LM/EM	Schönemann et al. (2013)
Syx15	GFP	Kissmehl et al. (2007)
SNAP 25 LP	GFP, immuno LM	Schilde et al. (2008)
H^+-ATPase		
c1, c4, c5 SUs	GFP	Wassmer et al. (2005, 2006, 2009)
F2	GFP	Wassmer et al. (2005, 2006, 2009)
a2	Immuno LM	Fok et al. (1995)
	GFP	Wassmer et al. (2006)
γ-Tubulin	Immuno LM	Klotz et al. (2003)
Acetylated tubulin	Immuno LM	Callen et al. (1994)
Calmodulin	Immuno LM/EM	Momayezi et al. (1986) Fok et al. (2008)
Ca^{2+}-release channels		
IP_3R	Immuno LM	Ladenburger et al. (2006)
Scaffolding proteins		
Stomatin1	Immuno LM	Reuter et al. (2013)
Stomatin4	Immuno LM	Reuter et al. (2013)

[a]For subcellular localization, see text.
[b]Only weak labeling.

Table 9.2 Components of contractile vacuole complex reported from other protists

Organisms	Component	Method	Reference
Tetrahymena	AP 2[a] paralog	GFP (pore)	Elde et al. (2005)
	RabD2	GFP	Bright et al. (2010)
	RabD10	GFP	Bright et al. (2010)
	RabD14	GFP	Bright et al. (2010)
	Calmodulin	pABs[b] (incl. pore)	Suzuki et al. (1982)
		pABs (incl. pore)	Numata and Gonda (2001)
	Centrin4	GFP (pore)	Stemm Wolf et al. (2005)
	Acetylated α tubulin	mABs[c]	Gaertig et al. (1995)
	Glutamylated tubulin	mABs	Wloga et al. (2008)
	γ Tubulin	HA tag[d] (pore)	Shang et al. (2002)
	NIMA related kinase[e]	GFP	Wloga et al. (2006)
Chlamydomonas	H^+ ATPase	pABs	Ruiz et al. (2001)
	H^+ PPase (pyrophosphatase)	pABs	Ruiz et al. (2001)
	TRP5 channels	Immuno LM	Fujiu et al. (2011)
	Exocyst components	GFP	Komsic Buchmann et al. (2012)
Amoeba proteus	H^+ ATPase	Immuno LM	Nishihara et al. (2008)
	ApAQP (aquaporin)	Immuno LM	Nishihara et al. (2008)
Dictyostelium[f]	PAT1 (a PMCA)	Immuno LM	Moniakis et al. (1995)
	Calmodulin	Immuno EM	Zhu et al. (1993)
	Copine A	GFP	Damer et al. (2005)
	Rh50	Immuno LM	Mercanti et al. (2006)
	P2X cation channels	Immuno LM	Fountain et al. (2007)
	Nramp/Slc11	GFP	Peracino et al. (2013)
	LvsA	Functional effects	Harris et al. (2002)

Table 9.2 Components of contractile vacuole complex reported from other protists—cont'd

Organisms	Component	Method	Reference
	H^+ ATPase, various SUs	Immuno LM/EM	Fok et al. (1993) Heuser et al. (1993) Nolta et al. (1993) Clarke et al. (2002)
	DAD 1 (cell adhesion molecule)	GFP	Sriskanthadevan et al. (2009)
	SecA (Munc18)	Functional	Zanchi et al. (2010)
	Exocyst	GFP	Essid et al. (2012)
	Clathrin and adaptor proteins		See text
	F BAR	GFP	Heath and Insall (2008)
	RabD	GFP	Harris and Cardelli (2002)
		pAB	Knetsch et al. (2001)
	Rab4	Immuno LM/EM	Bush et al. (1994)
	Rab8	GFP	Essid et al. (2012)
	Disgorgin (Rab8a GAP)	GFP	Du et al. (2008)
	Rab11	GFP	Harris et al. (2001)
	Rab14	Genomics	Bright et al. (2010)
	Drainin Rab11 effector	GFP	Becker et al. (1999) Essid et al. (2012)
	MEGAP (GAP)	GFP	Heath and Insall (2008)
	Rho GTPase modulator *Dd*RacGap1 (DRG)	pAB	Knetsch et al. (2001)
	Dajumin	RFP[g]	Gabriel et al. (1999)
		RFP[g]	Du et al. (2008)
		GFP	Jung et al. (2009)
	Tubulin	Immuno LM	Jung et al. (2009)
	MyoJ (myoV type)	Immuno LM	Jung et al. (2009)

Continued

Table 9.2 Components of contractile vacuole complex reported from other protists—cont'd

Organisms	Component	Method	Reference
Trypanosoma	H^+ PPase (pyrophosphatase)		Montalvetti et al. (2004)
	H^+ ATPase SU B	Proteomics[h]	Ulrich et al. (2011)
	Calmodulin	Proteomics	Ulrich et al. (2011)
	TcAQP (aquaporin)	Genomics	Montalvetti et al. (2004)
	Rab11	Proteomics	Ulrich et al. (2011)
	Rab32	Proteomics	Ulrich et al. (2011)
	AP 180	Proteomics	Ulrich et al. (2011)
	VAMP1 (v SNARE)	Proteomics	Ulrich et al. (2011)
	*Tc*SNARE2.1	Proteomics	Ulrich et al. (2011)
	*Tc*SNARE2.2	Proteomics	Ulrich et al. (2011)
	Disgorgin	Proteomics	Ulrich et al. (2011)
	Myosin	Proteomics	Ulrich et al. (2011)
	Clathrin heavy chain	Proteomics	Ulrich et al. (2011)
	$InsP_3R$/RyR related protein	Proteomics	Ulrich et al. (2011)

[a]AP-2, adaptor protein 2 (for clathrin coat assembly).
[b]pABs, polyclonal antibodies.
[c]mABs, monoclonal antibodies.
[d]HA, hemagglutinin.
[e]NIMA-related kinases are Ser/Thr kinases implicated in cell-cycle control.
[f]Some of the components listed have been shown to associate with the CVC of *Dictyostelium* only transiently, notably Disgorgin, LvsA, Rab8A, and Drainin (Du et al., 2008).
[g]Red fluorescent protein.
[h]Assignment to the CVC based on enriched fractions.

membrane to membrane interactions also outside the pore and the CV/ampullae connections. Their occurrence reflects the fusion capacity *in vitro* of vesicles derived from the *Paramecium* CVC (Sugino et al., 2005). *In vivo* fusion may serve for permanently ongoing biosynthesis by vesicle delivery. This also makes CVC resident *Pt*SNAREs relevant for maintaining Ca^{2+} homeostasis (Schönemann et al., 2013). Moreover, they may account for reversible fusion/fission processes within the spongiome for regulating membrane area during swelling and collapse of the CV and the radial arms during diastole/systole cycles (Schönemann et al., 2013). In

Dictyostelium, CVC resident SNAREs await characterization, but the SNARE associated protein SecA (=Munc18) has been found (Essid et al., 2012; Sriskanthadevan et al., 2009) to be mandatory for CV contents release (Zanchi et al., 2010). Three SNAREs—still to be characterized—have been assigned to the CVC in *Trypanosoma cruzi* (Ulrich et al., 2011), as specified in Table 9.2.

2.3.2 Exocyst, Rab-type GTPases, and additional regulators of vesicle trafficking

The multimeric exocyst tethers donor and acceptor membranes to each other (Guo et al., 2000; Heider and Munson, 2012). Next to join the intermembrane link, in concert with SNAREs, are Rab type monomeric GTP binding proteins (small G proteins or GTPases). Together with their regulators, such as GAP (GTPase activating proteins), GEF (guanine nucleotide exchange factor), and GDI (guanosine nucleotide dissociation inhibitor), they serve for improvement and acceleration of membrane specific interactions (Bustelo et al., 2007; Zerial and McBride, 2001). Several types have been found in the CVC of different protists, for example, *Tetrahymena* (Bright et al., 2010). In summary, the succession of steps are as follows: Multimeric protein complexes, called the exocyst, tether vesicles to a target membrane, followed by interaction with Rab GTPases and formation of a t (Q)/v (R) SNARE trans complex which mediates vesicle docking for subsequent fusion (Jahn and Fasshauer, 2012). The latter generally requires a very local Ca^{2+} signal (Neher, 2012).

Generally, the different Rab type small GTPases can be attributed to different sites and stages of intracellular membrane transport (Galvez et al., 2012; Stenmark, 2009; Zerial and McBride, 2001). Among the small GTPases found experimentally in the CVC of *Dictyostelium* are RabD (Harris and Cardelli, 2002; Knetsch et al., 2001), Rab4 (Bush et al., 1994, 1996), Rab8 (Essid et al., 2012), and Rab11 (Harris et al., 2001). (*Dd*RabD previously been considered identical with Rab4 is now considered rather close to Rab 14; see below.) RabD clearly regulates CV activity in *Dictyostelium*, in conjunction with *Dd*RacGap1, a protein containing both, Rho GEF and Rho GAP domains, similar to mammalian Bcr/Abr (Knetsch et al., 2001). Also in *Dictyostelium*, the RhoGDI homolog GD11 appears to be relevant for CV function (Rivero et al., 2002). The latter two proteins aim at Rho and Rac type GTPases, basically discussed in the context of actin organization; this is remarkable since no actin is known from the CVC (Section 5.1).

The near Golgi Rab11 is indicative of involvement of an endosomal recycling compartment in mammalian cells (Galvez et al., 2012; Takahashi et al., 2012). Its occurrence in the CVC of some species may support an emerging concept inferring the contribution of recycling processes although no further details are known. In *T. cruzi*, GTPases attributed to the CVC encompass not only Rab11 but also Rab32 (Ulrich et al., 2011). In *T. thermophila*, RabD2, RabD10, and RabD14 are restricted to the CVC (Bright et al., 2010). These *Tt*Rab proteins are lineage specific and thus different from Rab14 described for the CVC of *Dictyostelium* and from several related, but not yet localized Rabs in *Paramecium* (*Pt*31649, *Pt*47027, *Pt*53159, and *Pt*RabC49) (Bright et al., 2010). In *Dictyostelium*, some Rab effector and regulator proteins are also known from the CVC, that is, the Rab11 effector Drainin (Becker et al., 1999) and the Rab8a GAP Disgorgin (Du et al., 2008; see Section 4.2).

The occurrence of these Rab proteins in the CVC of different species underscores the importance of membrane trafficking in the CVC. Previously, this has been concluded from the occurrence of NSF (Kissmehl et al., 2002) and of SNAREs in the *Paramecium* CVC (Plattner, 2010a,b). Organelle specificity of both, Rabs and SNAREs supports evolution separately from other vesicular pathways, making the CVC an organelle newly "invented" during evolution.

2.3.3 Possible involvement of Golgi apparatus in CVC biogenesis

In *Dictyostelium*, RabD is now considered related to Rab14 (rather than to Rab4 as in earlier claims; Bush et al., 1996; Knetsch et al., 2001; Rivero et al., 2002) which in mammalian cells serves for Golgi → endosome transport (Junutula et al., 2004). This would go along with the importance of the clathrin adaptor protein, AP 1, a vesicle budding mediator operating in mammalian cells in the Golgi, for the biogenesis of the CVC in *Dictyostelium* (Essid et al., 2012; Lefkir et al., 2003). In *Tetrahymena*, the CVC harbors RabD subtypes D2 (around CV), D10 (on tubular extensions), and D14 (CVC associated large vesicles), whereas Rab4 is associated with the phagosomal system (Bright et al., 2010), thus supporting the lack of identity of Rab4 with RabD type GTPases.

The V0 part of the H^+ ATPase requires a glycoprotein, Voa1p in yeast, which drives its assembly (Ryan et al., 2008), before this complex dissociates for further transport to the Golgi apparatus. For the CVC, the pathway(s) of delivery is unknown. In the EM, with *Paramecium*, one can see a close association of the decorated spongiome with cisternae of the rough ER; besides

ribosomes, occasionally pegs (possibly H^+/V1 pegs) are recognized on the same ER cisternae (unpublished observation). Theoretically, a third path way for targeted delivery of H^+ ATPase SUs to the CVC could be via endo cytosis (Brown et al., 2009). In fact, the 100 kDa SU *vat*M in *Dictyostelium* is shared by both, the CVC and the endolysosomal membranes (Clarke et al., 2002). This pathway to the CVC is discussed in Section 5.2. Altogether, the pathway of H^+ ATPase constituents to the CVC probably occurs either from the ER (directly or via the Golgi apparatus) or via endocytosis, or by both mechanisms. In summary, the involvement of the Golgi in CVC biogenesis is poorly understood.

3. HANDLING OF CALCIUM BY CVC

The CVC of *Paramecium* has been shown to sequester and extrude Ca^{2+} and thus to contribute to intracellular calcium homeostasis. On the one hand, these cells are highly permeable to Ca^{2+}; on the other hand, as in any cell, intracellular concentration of free (dissolved) Ca^{2+}, $[Ca^{2+}]_i$, has to be kept low for the following reasons. Not only is too high a $[Ca^{2+}]_i$ level toxic, but low basic levels are also required for the use of Ca^{2+} as a second messenger. Later on, reflux of Ca^{2+} into the cytosol has also been documented, but nothing is known about any messenger effect in the CVC.

3.1. Calcium uptake by CVC

CVCs dispose of a primary active Ca^{2+} uptake mechanism only exception ally, for example, in *Dictyostelium*. Only a secondary active process based on primary H^+ uptake (H^+ pump) is universally distributed (Section 3.1.2).

3.1.1 Ca^{2+} sequestration by CVC

The CV of *Paramecium* releases substantial amounts of Ca^{2+} (Stock et al., 2002). This is based on chemiosmosis as it strictly depends on the ΔH^+ gen erated by the organellar H^+ ATPase. This is derived from the fact that con canamycin B, an efficient H^+ ATPase inhibitor in *Paramecium* (Grønlien et al., 2002), retards by about 10 fold the reestablishment of $[Ca^{2+}]_i$ homeo stasis after a significant Ca^{2+} load (Plattner et al., 2012).

No Ca^{2+} ATPase of the type sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase has been detected in the CVC of *Paramecium* (Hauser et al., 1998, 2000) and the same is true for the PMCA (plasma membrane Ca^{2+} ATPase) (Elwess and Van Houten, 1997). This differs from *Dictyostelium* where PAT1, a PMCA type Ca^{2+} pump, occurs also in the

CVC (Moniakis et al., 1995). However, PAT1 possesses no conserved auto inhibitory calmodulin binding domain (Pittman, 2011). Considering on the one hand that the potential calmodulin binding domain in the carboxy terminal part of orthodox PMCA molecules differs in *Dictyostelium* PAT1 (Moniakis et al., 1995) and on the other hand the wide variability of calmodulin binding sites, in general (Fraga et al., 2010), it remains open whether PAT1 has to be classified as a genuine or as an atypical PMCA. CaM binding studies could give the answer. See also Section 3.2.5 for the effect on anticalmodulin drugs on CV performance.

An alternative way of Ca^{2+} sequestration is reported from the CVC of *T. cruzi*, that is, a H^+ pyrophosphatase (H^+ PPase, Montalvetti et al., 2004). However, proteomics analysis of CVC enriched fractions also revealed H^+ ATPase SU B (Ulrich et al., 2011). In this parasite, the situation may be different insofar as its CVC is assumed to receive membrane components by fusion with acidocalcisomes whose H^+ PPase activity is well established (Docampo et al., 2005; Moreno and Docampo, 2009).

In summary, the H^+ ATPase can be considered the only primary active transporter in the CVC of *Paramecium*. For the additional PMCA type pump in *Dictyostelium*, it has to be analyzed to what extent it might support the organelle resident H^+ ATPase. The same is true of the H^+ PPase in *Trypanosoma*.

3.1.2 Calcium/proton exchangers and Aquaporin

In the absence of a Ca^{2+} pump in the CVC of ciliates, one must conclude that a secondary active Ca^{2+} transport is available, operating on the basis of the ΔH^+ generated by the H^+ ATPase. Though not yet identified in any CVC at a molecular level, such CAX (cation exchanger) molecules are found by genomics analysis in *Dictyostelium*, *Paramecium*, and Apicomplexa, the parasitic close relatives of ciliates (Shigaki et al., 2006). Functionally, such activity has been established, for example, in *Dictyostelium*, but assigned to acidic vesicles, addressed as "acidosomes" (Rooney and Gross, 1992). From experience with *Chlamydomonas*, this activity may be localized to acidocalcisome and/or to CV membranes, both endowed with H^+ pumps and Ca^{2+} sequestration activity (Ruiz et al., 2001). Since the CV of *Paramecium* also secretes Na^+, in addition to Ca^{2+} and Cl (Stock et al., 2002), several such transporters should be expected. In fact, in some systems different antiporters and Ca^{2+} pumps can cooperate (Orlowski and Grinstein, 2007). Considering the very likely occurrence of CAX molecules

also outside the CVC, structural localization is adviced. Aquaporin can also be assumed a general constituent of CVC membranes, although this postulate has been sufficiently verified only in *Amoeba proteus* (Nishihara et al., 2008) and the human pathogenic flagellate *T. cruzi* (Montalvetti et al., 2004).

3.2. Ca^{2+} release from the CVC by Ca^{2+}-channels

Several types of Ca^{2+} release channels (CRCs) are known from metazoan cells, but only quite recently the most important types have been identified in protozoa. Some of them are unambiguously, though unexpectedly, components of the CVC.

3.2.1 CRCs type inositol 1,4,5-trisphosphate and Ryanodine receptors

Sequestration of Ca^{2+} into the CVC makes the organelle also a pool of Ca^{2+} for backflow into the cytosol. In *Paramecium*, we registered spontaneous, transient, local Ca^{2+} signals (puffs) (Ladenburger et al., 2006). Concomitantly, CRCs typical of *P. tetraurelia* (*Pt*CRC) have been found in the CVC. One, designated *Pt*CRC II 1, has been identified as a genuine inositol 1,4,5 trisphosphate ($InsP_3$) receptor ($InsP_3R$) (Ladenburger et al., 2006). These $InsP_3Rs$ are distributed over the entire CVC except the decorated spongiome. Reflux of Ca^{2+} from the CVC via this constitutively active $InsP_3Rs$ into the cytosol can serve for fine tuning of cytosolic Ca^{2+} concentration. The CV also contains some additional CRCs (Ladenburger and Plattner, 2011). *Pt*CRC V 4 contains a rather short domain homologous to an $InsP_3$ binding domain (though this has not been tested experimentally) and is also rather broadly distributed over other organelles. Two other, closely related CRCs, *Pt*CRC VI 2 and *Pt*CRC VI 3 are devoid of an $InsP_3$ binding domain and show up near the CV pore (Ladenburger and Plattner, 2011). The occurrence of different *Pt*CRC paralogs in the CVC suggests functions in addition to $[Ca^{2+}]_i$ fine tuning, such as restructuring of the spongiome (see below).

3.2.2 Other types of Ca^{2+}-release channels

The *Paramecium* genomic database contains still other candidates for CRCs (Plattner et al., 2012). This includes two pore channels (TPC) and transient receptor potential channels. The activation of Ca^{2+} by microinjection of the two recently described channel activators (Lee, 2004), $NAADP^+$ (nicotinic acid adeninedinucleotide phosphate; Galione et al., 2010) and cADPR

(cyclic adenosine diphosphoribose, Zalk et al., 2007), affects the activity of the anterior CVC (Plattner et al., 2012). The CV reduces its pumping frequency by approximately ninefold, in response to microinjected cADPR (≤500 nM) or NAADP (≤100 nM) (Plattner et al., 2012). In both cases, for unknown reasons the anterior CV reacts strongly, in contrast to the posterior CV. This may be an indirect effect following the release of Ca^{2+} from other Ca^{2+} storage organelles. More work has to be done to clarify this intriguing aspect. Another uncertainty is the recent discovery that TPCs not always represent Ca^{2+} channels of acidic compartments (Patel et al., 2011), but that they may also be phosphoinositide activated Na^{+} channels in some mammalian cells (Wang et al., 2012). In *Chlamydomonas*, TRP5 is a component of the CVC (Fujiu et al., 2011).

The CVC of *Dictyostelium* contains an ATP gated ion channel type P2X, that is, a purinergic receptor type channel that can conduct Ca^{2+} and regulate CV activity under hypotonic conditions (Fountain et al., 2007; Sivaramakrishnan and Fountain, 2013). (To activate P2X channels one has to assume that ATP diffuses into the CV lumen.) Since one may also expect CRC type channels of the kind reported from *Paramecium* (Ladenburger and Plattner, 2011) in the CVC of other cells, the general question arises which may be the differential functions of different channels.

3.2.3 Ca^{2+}-release channels in CVC and local fusion processes

Any functional implications of the luxurious endowment of the *Paramecium* CVC with CRCs remain to be analyzed. Generally, a local $[Ca^{2+}]_i$ increase is a requirement for local membrane fusion processes. For several reasons discussed elsewhere (Schönemann et al., 2013), such sites may abundantly occur within the CVC. Evidently, fusions occur not only between the CV and the cell membrane but also between the CV and the ampullae (Section 2.3.1). However, membrane fusions must also take place during ongoing vesicle delivery for biogenesis and probably also for permanently ongoing restructuring processes within the smooth spongiome. In fact, silencing of some of the CVC SNAREs considerably impairs organelle function. (See Table 9.3 for the effects of gene silencing in *Paramecium*.) As outlined above, the entire CVC—except the decorated spongiome—is endowed with SNAREs. Regrettably, synaptotagmin, the Ca^{2+} sensor for fusion processes (Pang and Südhof, 2010), is not yet known from any of the protists; however, homologous molecules with a larger number of C2 domains do occur in *Paramecium* (Plattner et al., 2012).

Table 9.3 Effects of gene silencing on the CVC of *Paramecium*

Component	Effect	Reference
Actin		
Actin 9	~10 times prolonged contraction cycles	Sehring et al. (2007a)
Other actin isoforms	No effect	Sehring et al. (2007a)
SNARE-specific chaperone		
NSF	Reduced smooth and decorated spongiome formation (EM), prolon gated CVC cycles	Schönemann et al. (2013)
SNAREs		
Syb2	Slowed pulsation of CV CV swelling with high $[Ca^{2+}]_o$	Schönemann et al. (2013)
	Deformed cells with swelling of radial canals and prolongated CVC cycles	Schilde et al. (2006)
Syb6	CV swelling and reduced pulsation activity CV swelling with high $[Ca^{2+}]_o$	Schönemann et al. (2013)
Syx2	CV swelling and reduced pulsation activity CV swelling with high $[Ca^{2+}]_o$	Schönemann et al. (2013)
Syx6	Aberrant form and position of new CVCs	Schönemann et al. (2013)
Syx14	No effect with high $[Ca^{2+}]_o$, yet slowly[a] decreasing viability	Schönemann et al. (2013)
Syx15	No effect with high $[Ca^{2+}]_o$, yet immediate[b] loss of viability	Schönemann et al. (2013)
SNAP 25 LP	No clear effect	Schilde et al. (2008)
H^+-ATPase		
c2 or c4 or c6 SUs or combinations of c SUs	~5–10 times prolonged contraction cycles	Wassmer et al. (2005)

Continued

Table 9.3 Effects of gene silencing on the CVC of *Paramecium*—cont'd

Component	Effect	Reference
F SUs	Similar effect	Wassmer et al. (2005)
a2 SUs	CV swelling, cell death	Wassmer et al. (2006)
a3 SUs	No effect	Wassmer et al. (2006)
Stomatin4	Accelerated CV pumping	Reuter et al. (2013)

[a]Restricted viability within 30 min exposure to increased $[Ca^{2+}]_o$, from 1 to 5 mM.
[b]Immediate loss of viability within ≤2 min exposure to increased $[Ca^{2+}]_o$, from 1 to 5 mM.

3.2.4 Stomatin and mechanosensitive Ca^{2+} channels

Among a plethora of mechanosensitive ion channels are transient receptor potential (TRP) and Piezo type proteins (Delmas et al., 2011; Xiao and Xu, 2010). The *Paramecium* DB does contain orthologs of Piezo, mechanosensitive channels in mammals (Coste et al., 2010), as well as TRP channels, as summarized by Plattner et al. (2012). As known from other cells, some of the latter are sensitive to membrane stretch (Kung et al., 2010) and may occur partly in the cell membrane and partly in some organelles (Patel and Docampo, 2010).

The tension measured on the *Paramecium* CV (Sugino et al., 2005) is compatible with that required for activation of such channels (Kung et al., 2010). Mechanosensitive channels are likely to occur in the *Paramecium* CVC for the following reasons, although this remains hypothetic for the time being. (i) We find the scaffolding protein, Stomatin, subfamily 4 associated with the CVC (Reuter et al., 2013). Stomatin homologs are found from bacteria (Hinderhofer et al., 2009) up to man (Lapatsina et al., 2012), and they are known to serve for the positioning of mechanosensitive Ca^{2+} channels in a variety of systems, including Med1 in *C. elegans* where it associates with the Stomatin homolog Mec 2 (Huang et al., 1995). (ii) The *Paramecium* database contains a mechanosensitive Ca^{2+} channel type Piezo (Coste et al., 2010), but its char acterization and localization are still open. Ca^{2+} released by these or any other CRCs could contribute, for instance, to the rhythmic fusion processes

between ampullae and the CV as well between the CV and the cell membrane. (This would be different from the CRCs of the type $InsP_3R$ and RyR or mixed types thereof, as outlined above).

One of the basic aspects of Stomatin function in other systems is the implication of actin mediated effects on mechanosensitive ion channel activity, as derived from the coincidence of these components together with Mec 2 in *C. elegans* (Mannsfeldt et al., 1999). In the absence of any indication of CVC associated actin in *Paramecium*, it remains to be seen which mechanosensor occurs in this organelle. (However, see Section 5.1 for limitations in actin visualization.) Also, remarkable is the accelerated pumping frequency after silencing of *Pt*Sto4 (Reuter et al., 2013). So far it was possible only in *Chlamydomonas* to show the association of mechanosensitive channels type TRP5 with the region containing the CV (Fujiu et al., 2011), whereas other types were detected in other cell regions. Aquaporin, another component of the CVC—found in *A. proteus* (Nishihara et al., 2008) and to be expected in other systems (Section 3.1)—is also known to associate with Stomatin in human erythrocytes (Rungaldier et al., 2013). Again internal pressure could modify its activity, since human AQP1is known to close in response to membrane tension increments (Ozu et al., 2013).

3.2.5 Calmodulin

Calmodulin on the CV seems to be crucial for organelle function. The Ca^{2+} mobilizing effect of the anticalmodulin drug W7 on CV performance in *Dictyostelium* can be explained by its effect on LvsA binding to the CV (Malchow et al., 2006), a protein mandatory for CV function (Section 4.2). Similarly W7, calmidazolium, and trifluoperazine reduce the pulsation rate of the CV in *Tetrahymena* (Bergquist, 1989; Suzuki et al., 1982).

However, there is some discussion on the precise localization of calmodulin to the CVC in different species. The CV membrane in *Dictyostelium* displays bound calmodulin (Moniakis et al., 1995), but in EM immunolocalization studies, calmodulin is absent from the cytosolic side of the CV membrane in *Dictyostelium* (Zhu et al., 1993), as it is in *Paramecium* (Momayezi et al., 1986). Rather, by immuno EM labeling, calmodulin has been localized to the lumenal side of the CV of *Paramecium* (Momayezi et al., 1986) and *Dictyostelium* (Zhu et al., 1993). This corresponds to an extracellular localization. Whereas calmodulin is generally established as a cytosolic high affinity/low capacity Ca^{2+} binding protein (Cohen and Klee, 1988),

extracellular calmodulin is the subject of ongoing debates. Nevertheless, its occurrence is difficult to negate as it is released by and exerts specific effects on plant (Chen et al., 2004; Cui et al., 2005) and mammalian cells (Crocker et al., 1988; Houston et al., 1997). Extracellular calmodulin is also a regulator of chemotaxis in *Dictyostelium* (O'Day et al., 2012).

In the CV lumen, calmodulin might provide some regulatory feedback to ion channels although this has not been ascertained as yet. More recent EM studies with *Paramecium* revealed calmodulin along the microtubular cytoskeleton of the CVC (Fok et al., 2008). In *Tetrahymena*, calmodulin has been found in association with CV pore by immunofluorescence (Numata and Gonda, 2001; Suzuki et al., 1982). In summary, several details concerning calmodulin in the CVC remain unsettled.

4. UNIQUE STRUCTURAL ASPECTS AND MOLECULAR COMPONENTS

A membrane bounded organelle that periodically swells in diastole and shrinks in systole is quite unusual, particularly since biomembranes are not expandable. And what does "shrink" mean in the present context? How are CVC membranes transformed? In this section, we can also have a glimpse on molecules whose presence in the CVC is poorly understood.

4.1. What enables reversible organelle expansion and collapse?

In the LM, during diastole, the CV and the radial arm canals seem to reappear from nothing as they swell. However, these membranes cannot expand by stretching because, as a general rule, biomembranes cannot be subjected to expansion or compression by more than ~1% (Andersen and Koeppe, 2007; Mohandas and Evans, 1994). In reality, the swelling of CV and of radial arms (and ampullae, in *Paramecium*) during diastole takes place by reversing de tubularization of the membrane from a network into a smooth form (with some spongiome still remaining around the canals). This holds not only for *Paramecium* (Tominaga and Allen, 1998) but also for *Dictyostelium* (Heuser, 2006) and probably also for other species. This is an intriguing problem of membrane biology; particularly the revers ible branching of this membrane labyrinth is difficult to comprehend in terms of classical lipid bilayer structure. Alone tubularization requires special proteins (Shen et al., 2012). Only one candidate, F BAR

(FCH Bin amphiphysin Rvs) protein, is known from the CVC of *Dictyostelium* (Heath and Insall, 2008; see Section 4.2). Generally, BAR proteins serve for the formation of tubular extensions from planar membranes (Mim and Unger, 2012; Wu et al., 2010). Since these proteins are not known to cause tubule branching, as occurring in the CVC, possible mechanisms are discussed in Section 7. Prerequisite to all this transformation are mechanosensitive Ca^{2+} channels of the type described in Section 3.2, as they are required for sensing the pressure in the CV and for initiating contents release and organelle collapse. They may also generate a Ca^{2+} signal for membrane restructuring. All this remains to be analyzed in detail.

SNAREs may also be involved, for example, as trans complexes, in keeping spongiome tubules in dense packing independent of fusion processes. They may also initiate reversible fusion processes within the spongiome as occurring between the smooth and the decorated spongiome under hyperosmotic conditions (Ishida et al., 1996). Thus, de / reconnection of parts of the spongiome might be a regulation principle in response to changing physiological requirements.

4.2. CVC components known specifically from *Dictyostelium*

At this point, we recognize that many more molecular details are known from *Dictyostelium* (which is not true of all aspects).

4.2.1 Dajumin and MEGAPs

Dajumin has been defined as a standard marker for the CV in *Dictyostelium* (Gabriel et al., 1999) as it does not dissociate from the CV membrane (Du et al., 2008), in contrast to some of the proteins listed in Table 9.2. Its function remains to be established, and it looks as if no similar protein has been reported from other systems. In *Dictyostelium*, proteins of the MEGAP (mental retardation GTPase activating protein) group, type F BAR, associate with the forming tubules of the CVC whose formation they drive (Heath and Insall, 2008). Their inactivation in *Dictyostelium* delays pumping activity of the CV. For more details, see Section 4.1.

4.2.2 Cell adhesion molecule and Rh50 protein

The *Dictyostelium* CV is reported to contain a Ca^{2+} sensitive cell adhesion molelcule, *Dd*DAD 1 (Sriskanthadevan et al., 2009). This molecule is synthesized on free ribosomes and transported to the CV for insertion into the plasma membrane. There it may be transported by previous release in

vesicles inside the CV lumen. Accordingly, the CV of *Dictyostelium* would participate in an unconventional mode of secretion. In *Dictyostelium*, muta tional analysis of targeting of a membrane integrated CV specific protein, Rh50 (a homolog to the mammalian Rhesus protein), revealed the involve ment of a cluster of acidic amino acids as targeting motifs, and of clathrin and adaptor protein AP 1 (Mercanti et al., 2006).

4.2.3 Lvs proteins and Nramp/Slc11 protein

Lvs (large volume sphere) proteins are related to lysosomal trafficking reg ulator proteins identified in Beige mice whose mast cells display excessively large secretory lysosomes due to abnormal fusion processes. Of the six *Lvs* genes occurring in *D. discoideum*, LvsA localizes to the CV and is required for osmoregulation (Gerald et al., 2002). LvsA binds in a calmodulin dependent fashion (Malchow et al., 2006) and null cells display abnormal CV membranes (Harris et al., 2002). This has been interpreted as an indi cation of the involvement of the CVC in *Dictyostelium* in a recycling/ phagosomal activity (Harris et al., 2002), particularly also because expression of a dominant negative RabD GTPase alters phagocytic activity and mor phology of the CV network (Harris and Cardelli, 2002). Yet, this is in con trast to data discussed in Section 5.2.

Nramp (natural resistance associated macrophage protein) proteins are orthologs to mammalian Slc11 (solute carrier) proteins which are proton coupled transporters of divalent cations (Nevo and Nelson, 2006). Nramp1 mediates resistance to infection by *Legionella pneumophila*. Nramp2 is exclu sively localized to the CVC in *Dictyostelium* where it regulates iron homeo stasis and may serve for the storage of cations (Peracino et al., 2013).

4.2.4 Drainin and Disgorgin

Drainin, a peripheral membrane protein, is a homolog of proteins occurring from yeast to man. In *Dictyostelium*, where it has been discovered, it is essen tial for CV discharge by formation of the pore, probably by acting along a signaling cascade (Becker et al., 1999). Recent analyses identified Drainin as a Rab11a effector and indicate sequential recruitment of Drainin, Rab8a, and the exocyst (Essid et al., 2012). Drainin binding is paralleled by binding Disgorgin and followed by recruitment of LvsA (see above). Disgorgin, a GAP for Rab8a, is also required for CV content release by fusion with the cell membrane (Du et al., 2008). Disgorgin and LvsA, in concert with GTP hydrolysis by Rab8a, is also thought to initiate detachment of the empty CV after contents release (Essid et al., 2012).

5. CYTOSKELETAL ELEMENTS, MOTOR PROTEINS, ENDOCYTOTIC INPUT, AND CLATHRIN

Microtubules are dominant components of the CVC, whereas the presence of actin is generally denied. This may be premature, as we shall dis cuss. Also under debate is the role of membrane input by endocytosis and participation of clathrin.

5.1. Cytoskeletal components and motor proteins

Microtubules accompany the CVC from the pore to the end of radial canals in *Paramecium* (Allen, 2000; Schneider, 1960) as well as in *Tetrahymena* (Frankel, 2000). In ciliates, the microtubule cytoskeleton of the CVC shapes the organelle and its substructures. Their recognition by monoclonal anti bodies depends on the respective posttranslational modification (Adoutte et al., 1991) and only some of the commercial antibody types are successful (Wassmer et al., 2006). In *Dictyostelium*, CVC associated microtubules are much less evident. Here, no CVC staining is seen with antibodies recogniz ing the mitotic apparatus (Gabriel et al., 1999), but again a specific commer cial monoclonal antibody clearly reveals a microtubular system of the CVC (Jung et al., 2009).

At the pore, γ tubulin is found in *Tetrahymena* (Shang et al., 2002) and *Paramecium* (Klotz et al., 2003); it thus can serve for microtubule nucleation. More specifically, in *Tetrahymena,* the pore is also reported to contain glutamylated tubulin, that is, not only GLU γ tubulin (Shang et al., 2002) but also GLU α tubulin (Wloga et al., 2008). The relevance of this modification for CVC biogenesis and function remains to be settled.

In *Tetrahymena*, tubulin along radial arms is acetylated (Gaertig et al., 1995) as it is in *Paramecium* (Callen et al., 1994). In *Paramecium*, microtubules accompanying the CVC in full length also contain glutamylated tubulin (Bré et al., 1994). This microtubular scaffold of the CVC mediates its char acteristic star like shape in ciliates. It is unsettled whether in *Paramecium* such modifications mediate insensitivity of the CVC to nocodazole (Tani et al., 2000), although this is a most efficient drug in these cells (Pape et al., 1991). The plus→minus directed motor, dynein, is associated with the CVC microtubule system in *Paramecium* (Fok et al., 2008). There may be additional motor proteins at work to exert a pulling force to the radial arms also in opposite directions, as known from the endoplasmic reticulum.

No filamentous material, actin or centrin, is known to be associated with the CV of *Paramecium* (Allen, 2000) and F actin disrupting agents have no effect (Tani et al., 2000, 2002). Also for *Dictyostelium*, the absence of actin is reported (Heuser, 2006). Considering the failure to trace actin by affinity (Kersken et al., 1986) and immunolabeling in the CVC of *Paramecium* (Sehring et al., 2007a) and in *Dictyostelium* (Heuser, 2006), there is general agreement that the periodic contraction of the CV is not due to actin/myosin interaction (Allen, 2000; Heuser, 2006). May the CVC nev ertheless contain actin? Remarkably, proteins modulating Rac and Rho type GTPases are reported to be relevant for CV activity in *Dictyostelium* (Knetsch et al., 2001; Rivero et al., 2002). Both modulate actin dynamics, though this is not their exclusive function (Bustelo et al., 2007); for GTPase and modulators, see Section 2.3. Another intriguing detail is the finding of actin binding proteins in the CVC of *Tetrahymena* (Watanabe et al., 1998).

More stringent hints come from *P. tetraurelia*. Silencing of two, out of many isoforms of actin, had considerable effects. Silencing of *Pt*Act4 caused formation of multiple CVCs (Sehring et al., 2010). Silencing of *Pt*Act9 slowed down the CV pumping cycle (Sehring et al., 2007a) although in immunofluorescence this isoform was restricted to food vacuoles. In *Paramecium*, one might theoretically envisage very short polymers as they are found in the cortex of the related parasitic phylum, Apicomplexa (Gould et al., 2011). Also theoretically, such forms could escape detection and still participate in organelle specific functions. Remarkably, this actin isoform does not possess the typical binding sites for drugs causing polymer ization or depolymerization (Sehring et al., 2007b). Thus, they would nei ther bind standard affinity stains (phalloidin) nor react to filament degrading drugs (cytochalasins). As discussed in Section 7, the presence of actin, even in "cryptic" form, could be important for some specific functions. In fact, in *Dictyostelium*, where null mutants of the myosin V related MyoJ reveal its crucial role for CV docking, cytochalasin A produces phenocopies thereof (Jung et al., 2009).

Several myosin isoforms are associated with the CVC of *Dictyostelium*, notably myoV (Jung et al., 2009). Type V myosin, called MyoJ in *Dictyostelium*, is a bidirectional motor associated with the CVC microtubules in *Dictyostelium* (Jung et al., 2009). Therefore, microtubule associated motor proteins may serve for the maintenance of the extended shape of the CVC. Too little information is available from ciliates. Another aspect of myosin V in mammals is its binding to secretory organelles in coordination with Rab

and exocyst function, thus serving the secretory pathway (Donovan and Bretscher, 2012).

In summary, microtubules are important for organizing the CVC. The question of the presence and function of actin in the CVC is currently enigmatic, though this is supported by indirect evidence. Several motor proteins have been described, particularly in *Dictyostelium*, but this may not yet be the full inventory. Together with microtubules, motor proteins may contribute the characteristic star shape of the organelle in ciliates.

5.2. Endocytotic input and role of clathrin

No hints to endocytotic uptake of CV components after discharge have been found with fluorescent cell membrane dyes, neither with Cy3.5 in *Dictyostelium* (Gabriel et al., 1999) nor with the styrene stain FM1 43 in *Paramecium* (Klauke and Plattner, 2000). Can this be explained by the kiss and run type of fast fusion/fission coupling? Or by fluorescence quenching by luminal acidification? This should not play a role as the CV lumen is not remarkably acidic (Wassmer et al., 2009) because of permanent expenditure of H^+ ions. Remarkably, these results are in contrast to surface labeling experiments with *A. proteus* showing label transfer to the CV (Nishihara et al., 2008).

One suggestive argument in *Dictyostelium* could be the shared H^+ ATPase SUs in cell membrane and CV membrane (Clarke et al., 2002). In *Paramecium*, one could be tempted to speculate along two aspects. One is the simultaneous occurrence of *Pt*CRC V 4 in parasomal sacs (clathrin coated endocytosis sites) and in the CV (Ladenburger and Plattner, 2011). The other suspicious fact is the presence of *Pt*CRC VI 3 in terminal cisternae (early endosomes; Allen et al., 1992) and at the CV pore (Ladenburger and Plattner, 2011). In *Tetrahymena*, one may consider the occurrence of a paralog to adaptor protein AP 2 at the CV pore, as observed by expression as a GFP fusion protein (Elde et al., 2005), as a hint to an input by endocytosis. However, these colocalizations may be merely incidental particularly since most of these components, such as *Pt*CRCs, also occur at other sites of the cell.

The following arguments concerning an endocytotic input into the CVC are much more stringent and they have all been elaborated with *D. discoideum*. The evidence is based on the transport of CVC specific cargo and on specific sorting proteins. Specific cargo proteins are Dajumin, LvsA, and RH50, which are characterized in the Section 4.2. Sorting proteins encompass clathrin for vesicle budding from the cell surface of

Dictyostelium—remarkably independent of the adaptor protein AP 2 (Macro et al., 2012). (Note that in higher eukaryotes, AP 2 mediates the assembly of clathrin coated vesicles at the cell membrane in cooperation with several other proteins (Reider and Wendland, 2011).) This is in contrast to disturbed osmoregulation after knockout of AP 2 SUs (Wen et al., 2009). In *Dictyostelium*, knockouts of clathrin heavy (O'Halloran and Anderson, 1992) or light chains (Wang et al., 2003), of the adaptor protein AP 1 (Lefkir et al., 2003) or AP 180 (Stavrou and O'Halloran, 2006) each result in disturbed osmoregulation. Therefore, in *Dictyostelium*, clathrin based vesicular transport, be it from the Golgi apparatus or from the cell membrane, appears well documented.

The distribution of copine A (a C2 domain containing Ca^{2+} binding protein) in *Dictyostelium* over cell membrane, CV membrane as well as endosomal and phagosomal membranes (Damer et al., 2005) has also been considered as evidence of such input. Similarly seductive, but unreliable are some observations concerning an input by phagocytosis. As cited by Harris et al. (2002) "the CV network and phagocytosis have also been linked in *Tetrahymena pyriformis*. A 71 kDa protein, associated with the actin binding proteins, localized to both the CV and oral apparatus. . .suggesting that a connection may exist between the membranes involved for internalization and osmoregularity. . . (Watanabe et al., 1998)". However, the fact is that particles ingested by phagocytosis are never seen, for example, in the CV of *Paramecium*.

This can be summarized as follows. Whereas all this clearly documents the relevance of an endocytotic input into the CVC of *Dictyostelium*, no such data are available for ciliates. The same holds for the relevance of clathrin coated pits and vesicles. Never seen in the CVC, for example, of *Paramecium*, they show up nicely at the EM level in *Dictyostelium* (Heuser, 2006) where, by molecular tools, input via clathrin coated vesicles has been documented. Any possible input of membrane material into the CV by phagocytosis remains questionable.

6. THE CV PORE AND EPIGENETIC ASPECTS OF ORGANELLE POSITIONING

6.1. Components of the CV pore

The pore is the site where the CV fuses with the cell membrane in a kiss and run type exocytosis. In *Paramecium*, the site containing the pore is a ~1 μm wide depression where the CV membrane is intimately attached

to the cell membrane over a relatively large area, with a very narrow cyto plasmic seam in between (Allen, 2000). Some chemically still undefined material is seen in between (McKanna, 1973). As mentioned, microtubules emanate from the pore and extend to the tip of radial arms, whereas alveolar sacs are approaching only at a distance.

In *Dictyostelium*, the exocytotic process depends on Rab8 (Essid et al., 2012) and on *Dd*secA (Essid et al., 2012; Sriskanthadevan et al., 2009), a homolog of Munc18 which in mammalian cells interacts with SNAREs (Meijer et al., 2012; Zilly et al., 2006). By implication, SNAREs will par ticipate as will exocyst proteins—a postulate derived from work with *Chlamydomonas* (Komsic Buchmann et al., 2012). These components can be assumed to form part of the kiss and run fusion/fission machinery at the pore. From CV docking until discharge, a sequential attachment and detachment of a variety of additional proteins has been proposed, including Disgorgin, Drainin, and LvsA (Essid et al., 2012). MyoJ (a type V myosin) is also mandatory for CV docking in *Dictyostelium* (Jung et al., 2009).

In ciliates, a variety of components have been localized to the pore, yet all only at the LM level (Table 9.1). In *Tetrahymena*, these include adaptor pro tein AP 2 (Elde et al., 2005), calmodulin (Numata and Gonda, 2001; Suzuki et al., 1982), centrin4 (Stemm Wolf et al., 2005), a NIMA (never in mitosis A) kinase related protein kinase (Wloga et al., 2006), γ tubulin (Shang et al., 2002), and acetylated tubulin (Gaertig et al., 1995). Remarkably, in mam malian neurons, NIMA family kinases are relevant for microtubule organi zation (Chang et al., 2009).

In *Paramecium*, pore associated proteins include calmodulin (Fok et al., 2008), the SNARE chaperone NSF (Kissmehl et al., 2002), SNAREs type Syb2 (Schilde et al., 2006), Syx2 (Kissmehl et al., 2007), Syb9, and Syx15 (Schönemann et al., 2013); the latter two, however, also occur over the rest of the CVC (except the decorated spongiome; Schönemann et al., 2013). In addition, CRCs types *Pt*CRC VI 2 and *Pt*CRC VI 3 are observed here (Ladenburger and Plattner, 2011); however, *Pt*CRC VI 2 is also present on ill defined cortical vesicles outside the CVC and *Pt*CRC VI 3 at the terminal cisternae (considered as early endosomes; Allen et al., 1992). Stomatins of type 1 and 4 are also localized to the CVC pore in *Paramecium* (Reuter et al., 2013). This is interesting as Stomatin is the only member of the stomatin–prohibitin–flotillin/reggie–HflC/K family currently known from protozoa and since Stomatin is associated predominantly, if not exclu sively, with mechanosensitive Ca^{2+} influx channels (Lapatsina et al., 2012). Therefore, the pore may have a sensor for the filling state of the CV—a

hitherto hypothetical aspect to be scrutinized. As in other exocytotic processes (Pang and Südhof, 2010), any of these channels may provide Ca^{2+} for the extrusion of vacuole contents at the pore.

The remarkable input of data from *Tetrahymena* is in part due to expression as GFP fusion proteins which otherwise would have remained undetected. Therefore, these components may be assumed to have a broader distribution among CVCs, also in other species. This may also be the case with SNAREs, CRCs, and Stomatins which so far have been identified and localized to the pore only in *Paramecium*.

6.2. Biogenesis and epigenetically determined positioning of CVC in *Paramecium*

New CVCs are formed anterior to each of the two CVC before cells undergo cytokinesis, their positioning being under epigenetic control in *Tetrahymena* (Frankel, 2000; Nanney, 1966) and *Paramecium* (Beisson, 2008; Klotz et al., 2003). It appeared to us most attractive to study the relevance of specific CVC proteins for biogenesis by inducing *de novo* formation. We tried the method elaborated by Iwamoto et al. (2003) to induce formation of supernumerary CVCs in sterile *P. multimicronucleatum* by exposure to a hypertonic medium with increased $[Ca^{2+}]_o$. Unfortunately, this method—although well reproducible under the same culture conditions—proved unsuccessful with *P. tetraurelia* cultures which were raised for gene silencing by feeding with transformed bacteria. These contained specific nucleotide sequences encoding SNAREs (Schönemann et al., 2013) and other CVC components to be silenced, with the aim to study the relevance of specific components for biogenesis. For these practical reasons, we have been restricted to register occasional effects on organelle biogenesis under asynchronous conditions (Table 9.3).

Silencing of different membrane components in *Paramecium* cultures revealed that the organelle resident v and t SNAREs are important to a different extent (Schönemann et al., 2013). Silencing of some of them makes the system particularly sensitive to increased $[Ca^{2+}]$ in the medium. The organelle was also impaired after silencing the IP_3R or of some SUs of the organelle resident H^+ ATPase (Schönemann et al., 2013). This might be due to a remote effect as silencing of the IP_3R also affects other functions; this is concluded from the substantial reduction of the biogenesis of secretory organelles (trichocysts) (Ladenburger et al., 2006). For unknown reasons, expression of a a2a3 chimera of the H^+ ATPase can cause swelling of the CVC or formation of supernumerary organelles (Wassmer et al., 2006).

Similarly, silencing of Syb2 1 or Syb2 2 isoforms causes formation of additional CVCs in some cells which also display a grossly deformed morphology (Schilde et al., 2006). Therefore, the overall cell surface pattern appears important. *De novo* formation of supernumerary CVCs, combined with malpositioning, has been most frequently observed after Syx6 silencing—the SNARE with a most dramatic effect on organelle performance (Schönemann et al., 2013). All this suggests that the CVC is an intriguingly cooperative system, with multiple feedback phenomena and with multiple cues controlling epigenetic positioning of CVCs in *Paramecium*.

In *Tetrahymena*, a precise geometrical arrangement of extended cortical structures determines the actual position of the CVC (Nanney, 1966). The molecules or stimuli (or local inhibitors) enabling proper placement of *de novo* forming CVCs are not known. Also in *Paramecium*, CVC docking sites are stereotypically arranged, the microtubules of one of the radial arms being in contact with the analogous structure of the old organelle (Allen et al., 1990). Then, all arms reach about the same length. May the steady state equilibrium between ongoing polymerization at the new CVC, with γ tubulin as a crucial nucleator at the pore (Klotz et al., 2003; Shang et al., 2002), and depolymerization at the periphery of the old CVC play a role? If so, what regulates the positioning of γ tubulin?

Do phosphorylation/dephosphorylation processes contribute to proper positioning? The relevance of phosphorylation processes has been assumed for general surface pattern formation in *Paramecium* where distinct phosphoproteins are localized to microtubule organizing centers (Sperling et al., 1991), centrin being a candidate (Klotz et al., 1997). After expression as a GFP fusion protein, centrin has been observed at the CVC pore in *Tetrahymena* (Stemm Wolf et al., 2005). Since calmodulin occurs at the pore (Numata and Gonda, 2001; Suzuki et al., 1982), calmodulin and calmodulin dependent enzymes may act as regulators (possibly also CDPK type kinases with integrated calmodulin motifs which may be recognized by anticalmodulin antibodies). Some additional questions arise, for example, whether the CRCs found at the CVC pore of *Paramecium*, *Pt*CRCVI 2 and *Pt*CRC VI 3 (Ladenburger and Plattner, 2011), may play any role in signaling for biogenesis. In summary, many details remain unexplained at this time.

Another line of search may be suggested in consequence of the finding of NIMA related kinases, after overexpression as a GFP fusion protein, at the pore of *Tetrahymena* (Wloga et al., 2006). These kinases associate with microtubule organizing centers not only of the mitotic spindle (O'Regan

et al., 2007). As shown with neurons, it also associates with cytoplasmic microtubules containing acetylated tubulin (Chang et al., 2009), as it occurs at the CVC pore (Gaertig et al., 1995). All these hypothetic considerations may be relevant for the epigenetically controlled positioning of the CVC.

7. CONCLUSIONS AND HYPOTHESES

Different types of CVC proteins have been analyzed in the different systems in widely different detail. This also encompasses proteins of vital importance for organelle function. Therefore, it appears justified in this final section to ask for likely general features and components. The aim is not only to establish a basic structural and functional feature of the organelle—even if hypothetic—but also to suggest an outline for further scrutiny in systems lagging behind.

7.1. Summary of a molecular anatomy of CVC

The CVC shows a similar principle of construction and activity in different freshwater protists, but it displays a most regular form in ciliates. Typically, the CVC is made up of a CV to which, in ciliates, radial arms (collecting canals) are attached in a most regular form. From there a tubular network emerges (spongiome). The spongiome consists of the tubular network of the smooth spongiome which is in continuity with the more distal decorated spongiome that is studded with H^+ ATPase molecules. For the decorated spongiome, Allen (1995) has proposed a tubulation effect of H^+ ATPase/pump molecules which are connected to each other by molecular links. From the smooth spongiome, the sequestered fluid reaches the radial arms (collecting canals), the ampullae, and the CV. Ampullae fuse and disconnect in each activity cycle, as does the CV with the cell membrane. Although there is some variation in the structure of the CVC in different species, they all share these essential features.

The H^+ ATPase generates a ΔH^+, thus energizing the membranes for chemiosmotic activity linked to the uptake of water and ions, including Ca^{2+}. However, other H^+ pumps can also occur in some systems. Also, pri mary active Ca^{2+} transport is no general feature of CVCs.

A variety of proteins (SNAREs, Rab GTPases), serving for membrane to membrane interactions including fusion, are known predominantly from ciliates. In *Paramecium*, we currently know of seven types of CVC resident SNAREs, each three v /R and t /Q SNAREs and one Qab SNARE. SNAREs are complemented by exocyst components, though analyzed only

partially in some species. In *Paramecium*, different CRCs of the type RyR and $InsP_3R$ are localized to the CVC, where they serve for partial Ca^{2+} reflux. No CRCs of comparable type are reported from other systems, but there is evidence of some other Ca^{2+} channels including TRP and P2X type channels. The occurrence of mechanosensitive Ca^{2+} channels is inferred from the presence of Stomatin in *Paramecium* and from the peri odic activity depending on internal pressure. Ca^{2+}/H^+ and possibly other antiporters have to be postulated for organelle function. The same holds for aquaporins which have been ascertained only rarely for a CVC. Based on silencing experiments, the importance of a variety of components for CVC function in *Paramecium* is presented in Table 9.3.

In *Dictyostelium*, also one of the most intensely analyzed systems, a variety of unexpected membrane proteins were detected in the CVC (Section 4.2), such as MEGAP, Lvs, and PAT1, a PMCA type Ca^{2+} pump. Also in *Dictyostelium*, in agreement with vesicle trafficking, different Rab type pro teins and Rab effectors and modulators, such as Drainin and Disgorgin, have been found. Lineage specific Rabs are localized to the CVC in *Tetrahymena* and in *Paramecium*. In a proteomics analysis, evidence of SNAREs and Rabs has also been found in the CVC of *T. cruzi*.

A microtubule system containing posttranslationally modified tubulin maintains the shape of the CVC in amoebocytes and ciliates. Considerable molecular information about microtubules and components of the pore come from *Tetrahymena*. Different types of myosin, but no actin, have occa sionally been reported. How silencing of specific actin isoforms in *Parame-cium* affects biogenesis (*Pt*act4) and function (*Pt*act9) remains unsettled. The bidirectionally active myosinJ in *Dictyostelium* (Jung et al., 2009) may serve for *in situ* expansion of the CVC arms along the supporting microtubules. In principle, this finding may be of relevance also for other systems where dynein has been found (*Paramecium*).

7.2. Generalized scheme of CVC function

One may imagine the following scenario. The central role of a ΔH^+ is para lleled by water and ion extrusion, supported by aquaporin and cation antiporters. This leads to CV swelling which is sensed by mechanosensitive Ca^{2+} channels (though still hypothetic for any of the CVCs) positioned at strategic sites. By subsequent signaling steps, membrane fusion between the CV and the plasma membrane (in ciliates also between CV and radial arms) is induced. This scenario is supported by two observations in *Paramecium*.

(i) Stomatin proteins, potential scaffolds for positioning mechanosensitive channels, are found in the CVC. (ii) The extrusion site (pore) contains par ticular isoforms of SNAREs and CRCs.

Internal restructuring from a branched tubular to a planar configuration and reverse, as occurring during systole/diastole cycles, can be produced by F BAR proteins (known so far only from *Dictyostelium*). More difficult to understand is the mechanism of branching and debranching of spongiome tubules. The process of branching/debranching may adjust the spongiome membrane area for regulatory processes to the actual functional require ments. It will require an extra set of CVC resident proteins which may include SNAREs and CRCs for reversible fusion/fission processes. This hypothetic function would be beyond their indisputable role for the delivery of membrane proteins by vesicles. In fact, different SNAREs are all scattered over the entire smooth spongiome in *Paramecium* (Kissmehl et al., 2007; Schilde et al., 2006; Schönemann et al., 2013). Reversibly branched tubules may alternatively be formed by proteins similar to those known from the ER which also possesses a tubulovesiclular organization. Among them are, in the ER, members of the Reticulon superfamily (Friedman and Voeltz, 2011) in conjunction with a GTPase termed atlastin, as summarized by (Daumke and Praefcke, 2011). Atlastin proteins are related to large GTPases of the type dynamin (Byrnes et al., 2013) which in turn could cause fission—again hypothetically, if present. It has to be emphasized that, with the exception of SNAREs and CRCs, none of these proteins are established components of the smooth spongiome, but for F BAR proteins this is rather likely. Clearly, the reversible formation of branched tubules in the smooth spongiome is currently difficult to understand and requires analysis. The same holds for the possible stabilization of the densely packed tubule system by trans SNARE complexes.

7.3. Steady-state biogenesis by vesicle trafficking and protein turnover

Steady state turnover of organelle components requires protein delivery by vesicle trafficking although no vesicle trafficking is directly seen in the EM. This cryptic biogenesis serves for replacement of molecular components for which SNAREs, exocyst complexes, and Rab proteins are required. For vesicle delivery, local [Ca^{2+}] provided by some of the organelle resident CRCs would play a role. Some components may be jointly delivered and some may reach the CVC after synthesis on free ribosomes. The involve ment of the Golgi apparatus is very likely, but not analyzed in detail. Many

details are not yet known, for example, the role of the different CRCs and which cargo proteins ride with one and the same vesicle, which ones are Golgi derived, and which membrane proteins are delivered by free ribo somes. Input by endocytotic components is well founded up to now mostly in *Dictyostelium*. Evidence comes from the presence of adaptor proteins and, also in *Dictyostelium,* smaller vesicles fusing to a big CV are considered of endocytotic origin (Macro et al., 2012; Zanchi et al., 2010). Differences in the endocytotic input may exist between the different systems.

Microtubule bundles associated with the fully developed CVC serve for reinforcement, rather than as trafficking rails since they are surrounded by the densely packed spongiome. These microtubules may require the post translational modifications reported (Section 5.1) to achieve stability and interaction with motor proteins. Among them are myosins and dyneins, but kinesins also have to be expected.

Not only silencing of SNAREs reduces the spongiome in the *Paramecium* CVC, as shown by Schönemann et al. (2013) for Syx2 and in Fig. 9.3 for Syb2, but also silencing of *Pt*CRC II/InsP_3R (Fig. 9.4). Also simultaneous silencing of H^+ ATPase SUs c2, c4, and c6 (in experimental series described by Wassmer et al., 2006) results in similar changes in ultrastructure. This

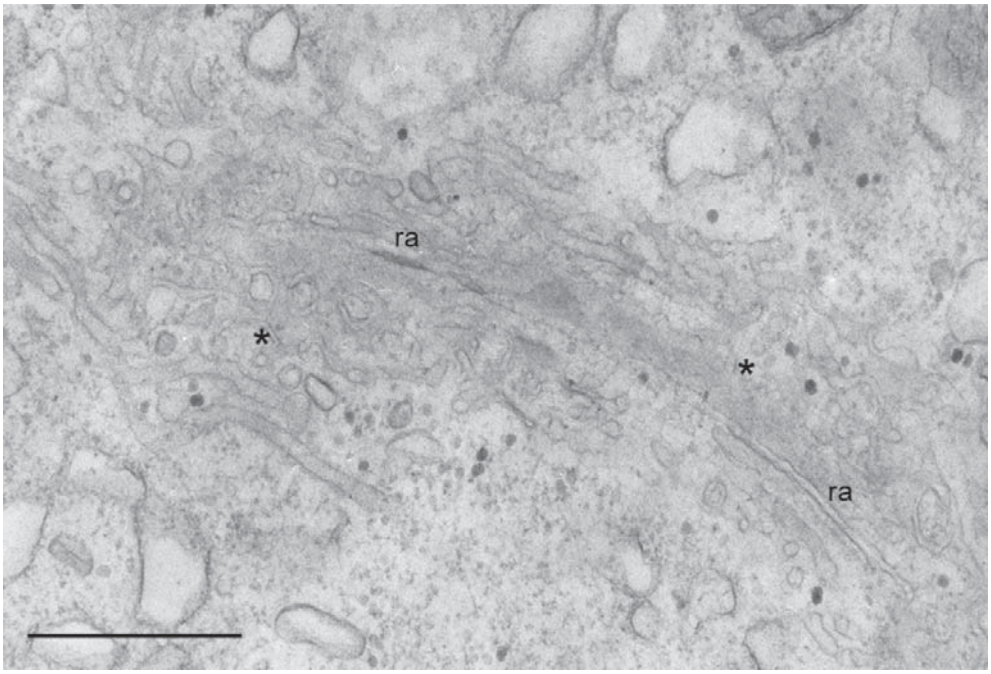

Figure 9.3 Degradation of CVC ultrastructure in *P. tetraurelia* after silencing of Syb2. To be compared with the control presented in Fig. 9.2. The radial arm (ra) is collapsed and only vestigial remnants of the smooth and decorated spongiome are left (asterisks). Bar = 0.1 μm. *Unpublished micrograph from experimental series described by Schilde et al. (2006).*

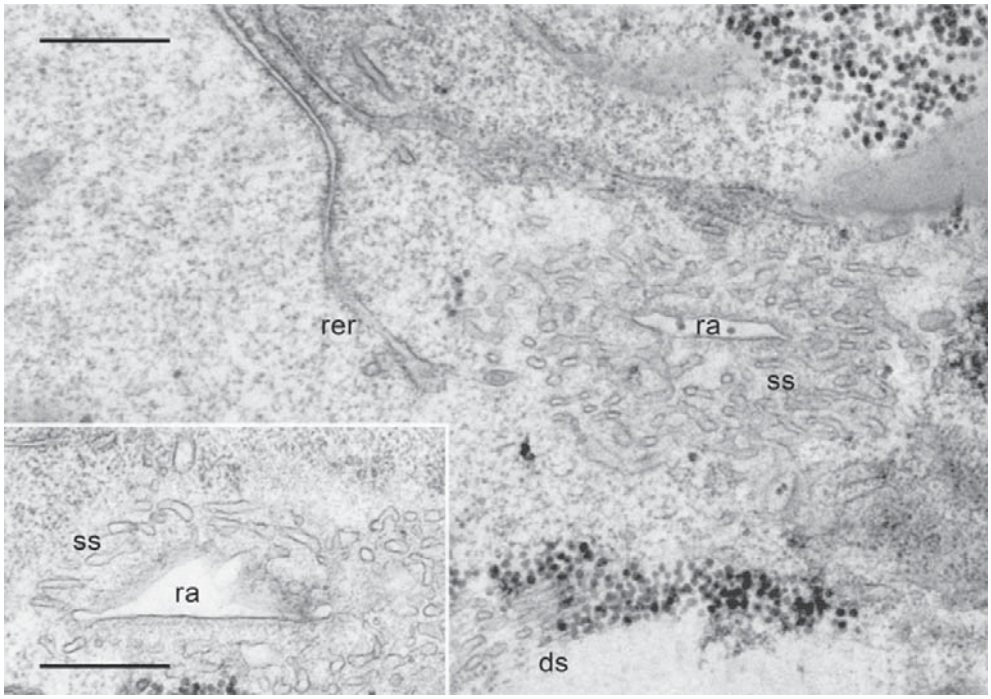

Figure 9.4 Silencing of CRC-II/InsP_3R in *P. tetraurelia* causes degradation of the CVC. Note the unusually loose packing of the smooth spongiome (ss) around a radial arm (ra). Remnants of the decorated spongiome (ds) are recognized only at a distance from the smooth spongiome. As in a normal cell (Fig. 9.2), some cisternae of the rough ER (rer), partially devoid of ribosomes, approach the CVC. Inset: Further degradation of the smooth spongiome around a radial arm which, on its lower part, is kept in shape by a microtubule support. Again cisternae of the rough ER approach the CVC. Bars = 0.1 μm. *Unpublished micrographs from experimental series described by Ladenburger et al. (2006).*

indicates mutual dependency of the delivery of CVC resident proteins—an interplay between fusion capacity, Ca^{2+} regulation, and H^+ sequestration as the basic primary function of the CVC.

7.4. Hypothetic considerations about *de novo* CVC biogenesis

During *de novo* biogenesis in *ciliates*, the CVC is placed at predictable sites, and several scenarios can be discussed. Since both of the new CVCs always assemble at defined sites of the cell surface and in strict relationship to the inherited organelles, this speaks for an epigenetic control by morphogenetic factors. Its formation/expression may depend on the context of the defined geometrical arrangement of the cortex at a certain distance in anterior direc tion from the old CVCs. In analogy to multicellular systems, control may follow the Gierer–Meinhardt model (Gierer and Meinhardt, 1972; Meinhardt, 2006)—hypothesis (i)—involving unknown soluble factors; these may operate by an antagonism of stimulatory and/or inhibitory effects.

Alternatively one may consider the relevance of firmly installed CVC protein components (hypothesis (ii)). One may assume such a role for proteins occurring at the CV/plasma membrane interface, that is, the pore. In a first step, a new pore would have to be assembled in order to serve as a nucleation site for a young CV. This hypothesis considers two facts, that is, (i) a new CVC forms around a small CV as a core part from which subsequently radial arms grow out. (ii) A new CVC forms in contact with the end of one of the radial arms of an old CVC (Allen et al., 1990). Again a dual question emerges: Are there special proteins at the pore and at the tip of a radial arm of a preexisting CVC, respectively? Remarkably, the radial canals of the old CVC shrink, while the new ones expand (Allen et al., 1990).

This suggests a steady state balancing, once the nucleation site for a new CVC has been established. The pore is known to contain γ tubulin, and possibly also centrin, as a nucleation center; see Section 5.1. Just like at other sites of the cell, such components of microtubule organizing centers can represent only one point in a chain of molecular components and their stepwise arrangement and eventual modification. In *Paramecium*, dynamic cell surface structuring depends on the phosphorylation state of different cortical proteins (Keryer et al., 1987; Sperling et al., 1991) including centrin (Klotz et al., 1997). In fact, centrin4 has been found at the pore of *Tetrahymena* by Stemm Wolf et al. (2005) (although in non dividing *Tetrahymena* centrin has not been found at the pore by Elde et al., 2005). Generally, centrin is associated with microtubule organizing centers (Levy et al., 1996) and is a target for reversible phosphorylation processes (Thissen et al., 2009). Yet, in the only study the effect of a general kinase inhibitor on the CVC in *Paramecium* has been classified as "partly normal" (Kaczanowska et al., 1996). More work is desirable.

As to regulation of microtubule length at their plus end, several proteins are to be envisaged. Considering that some kinesin superfamily members can exert dual functions, it appears rewarding to look for microtubule associated motor proteins. For instance, Kif18A restricts the length of kinetochore microtubules of the mammalian cytospindle (Mayr et al., 2007), and Kif19A regulates the length of cytoplasmic microtubules in mammalian cells (Niwa et al., 2012). If such a mechanism could be found with CVC associated microtubules, this would be relevant not only for defining the final size of CVC arms but also for formation of new ones adjacent to the end of an old radial arm. The protein kinase NIMA, known to regulate the length of axonemal microtubules and to occur at the pore of the *Tetrahymena* CV (Wloga et al., 2006), could also spring into action. Local posttranslational modifications of tubulin should also be envisaged.

From another point of view, it may be feasible to compare the *de novo* biogenesis of the CVC with that of cilia and flagella. Both organelles are separated by a membrane from the outside medium (considering that the lumen CVC periodically opens to the outside), the outside/luminal medium is rich in Ca^{2+} (CV; Stock et al., 2002) and not remarkably acidic (CV according to stains undergoing protonation; Wassmer et al., 2009), and both organelles are supported by microtubules with antero and retrograde motor proteins attached (for cilia and flagella, see Ishikawa and Marshall, 2011). This suggests the potential relevance of similar factors for the regulation of organelle biogenesis. Considering that *de novo* genesis of the CVC starts before cell replication, phosphorylation processes may be crucial, just as in cilia. Thereby, cyclin dependent kinases (Tam et al., 2007) and MAP (mitogen activated protein) kinase (Berman et al., 2003) act as regulators, as do other phosphorylation processes and kinases (Besschetnova et al., 2010). Probably, the level of local $[Ca^{2+}]_i$ also plays a role in the biogenesis of cilia and flagella (Besschetnova et al., 2010; Wemmer and Marshall, 2007). The latter may be controlled by any of the CVC resident CRCs (beyond the fine tuning effect described for *Pt*CRC II/$InsP_3R$ in Section 3.2.1). Depending on local $[Ca^{2+}]_i$, the insertion of new membrane could thus be regulated. Cytosolic levels of soluble tubulin are also a factor regulating ciliary length (Sharma et al., 2011). At this point, a depolymerizing Kif18 activity may act as a switch between the old and the new CVC during *de novo* formation.

The expansion of scaffolding microtubules from the nucleation site of a newly forming CVC would be paralleled by formation of radial canals and of the spongiome by vesicle delivery. EM images obtained after silencing dif ferent CVC membrane proteins show unilateral association of membranes (Fig. 9.4, inset), thus suggesting this sequence for *de novo* biogenesis.

In summary, both soluble factors and/or insoluble structural components may be relevant for the regulation of *de novo* CVC biogenesis. One can now consider several lines of thoughts that are all amenable to experimental analysis.

7.5. Complexity of protein pattern to be expected in future research

Together, from all systems analyzed, one may currently sum up the number of proteins identified in the CVC to nearly about 100, some in multimeric complexes. Considering the complexity of the organelle several times, more proteins may reasonably be expected.

ACKNOWLEDGMENT

The author's work cited herein has been supported over the years by the German Research Council.

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