

When is a cell not a cell? A theory relating coenocytic structure to the unusual electrophysiology of *Ventricaria ventricosa* (*Valonia ventricosa*)

Review article

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Summary. *Ventricaria ventricosa* and its relatives have intrigued cell biologists and electrophysiologists for over a hundred years. Historically, electrophysiologists have regarded *V. ventricosa* as a large single plant cell with unusual characteristics including a small and positive vacuole-to-outside membrane potential difference. However, *V. ventricosa* has a coenocytic construction, with an alveolate cytoplasm interpenetrated by a complex vacuole containing sulphated polysaccharides. We present a theory relating the coenocytic structure to the unusual electrophysiology of *V. ventricosa*. The alveolate cytoplasm of *V. ventricosa* consists of a collective of uninucleate cytoplasmic domains interconnected by fine cytoplasmic strands containing microtubules. The cytoplasm is capable of disassociating into single cytoplasmic domains or aggregations of domains that can regenerate new coenocytes. The cytoplasmic domains are enclosed by outer (apical) and inner (basolateral) faces of a communal membrane with polarised K⁺-transporting functions, stabilised by microtubules and resembling a tissue such as a polarised epithelium. There is evidence for membrane trafficking through endocytosis and exocytosis and so “plasmalemma” and “tonoplast” do not have fixed identities. Intra- and extracellular polysaccharide mucilage has effects on electrophysiology through reducing the activity of water and through ion exchange. The vacuole-to-outside potential difference, at which the cell membrane conductance is maximal, reverses its sign from positive under hypertonic conditions to negative under hypotonic conditions. The marked mirror symmetry of the characteristics of current as a function of voltage and conductance as a function of voltage is interpreted as a feature of the communal membrane with polarised K⁺ transport. The complex inhomogeneous structure of the cytoplasm places in doubt previous measurements of cytoplasm-to-outside potential difference.

Keywords: *Ventricaria ventricosa*; Cytoplasmic structure; Vacuolar structure; Sulphated polysaccharide; Coenocyte; Algal electrophysiology.

Abbreviations: PD_{co} cytoplasm-to-outside potential difference; PD_{vo} vacuole-to-outside potential difference; PD_{vc} vacuole-to-cytoplasm potential difference; I/V current as function of voltage; G/V conductance as function of voltage.

Cell biology and electrophysiology of *Ventricaria ventricosa*: a hundred years of research

The marine alga *Ventricaria ventricosa* (Olsen and West 1988; formerly *Valonia ventricosa*) lives in coral rubble in tropical reef environments such as the Great Barrier Reef. The giant-celled *V. ventricosa* and related algae of the order Cladophorales have fascinated cell biologists and electrophysiologists alike since the early twentieth century.

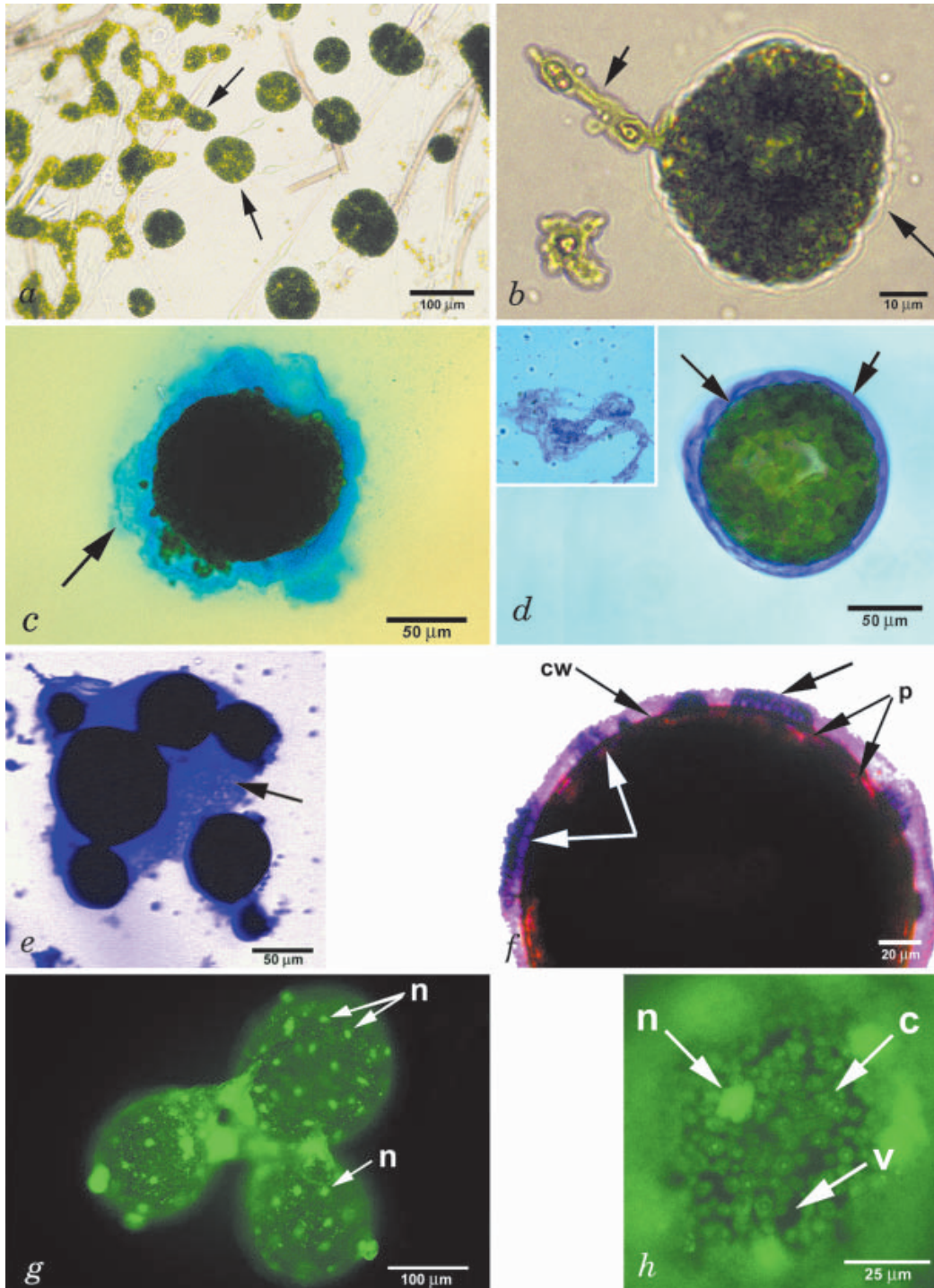
Some members of the Cladophorales respond to injury with the extraordinary process of modified segregative cell division (for reviews, see La Claire 1982, Menzel 1988). The entire cytoplasm of a punctured or cut cell contracts into a network and after about 30 min forms inwardly swollen cytoplasts connected by thin strands of cytoplasm. After 1–3 h the cytoplasmic strands are severed leaving numerous separate protoplasts (aplanospores) that then regenerate cell walls. This process has been studied since the 1930s, in *V. ventricosa* (Kopac 1933, Doyle 1935, Steward and Martin 1937, La Claire 1982, Nawata et al. 1993) and in *Boergesenia forbesii* (Enomoto and Hirose 1972; La Claire 1982; O’Neil and La Claire 1984, 1988; Itoh et al. 1984). Protoplast formation in *V. ventricosa* is illustrated in Fig. 1a and b. The ability to regenerate new cells from frag-

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ments of cytoplasm distinguishes *V. ventricosa* and its relatives from most other organisms.

Ventricaria ventricosa has played a key role in plant cell electrophysiology and continues to do so. Osterhout's group made electrical measurements of *V. ventricosa* in the 1920s that helped establish the concept of a transmembrane electrical potential difference (PD) in plant cells

(Hope and Walker 1975). However, the small positive values measured for the vacuole-to-outside potential difference (PD_{vo}) then and in the 1930s (e.g., +15 mV; Blinks 1930, Steward and Martin 1937), later turned out to be highly unusual. Most plant cells have a negative PD_{vo} , which is dominated by a highly negative cytoplasm-to-outside PD (PD_{co}) (-230 to -280 mV in *Chara corallina*)



and which is only slightly offset by a small vacuole-to-cytoplasm PD (PD_{vc}) (about +20 mV in *C. corallina*). There have been many attempts to reconcile the unusual electrical properties of *V. ventricosa* with conventional ideas of the structure of plant cells. *Ventricaria ventricosa* is regarded in the electrophysiological literature as a single spherical cell containing a tonoplast-enclosed central vacuole and a thin layer of plasmalemma-bound cytoplasm. However, the “cells” have a coenocytic construction and are extraordinarily large, with some up to 10 cm in diameter (Menzel 1988). The possibility that the coenocytic structure might be coupled to the unusual electrophysiology has not previously been considered.

Ventricaria ventricosa, a coenocyte with unusual cytoplasmic and vacuolar structure

The cytoplasm of *V. ventricosa* has an unusual and complex organisation. The electron micrographs of Shihira-Ishikawa and Nawata (1992) show a remarkable alveolate or “sponge-like” cytoplasmic topology. Most of the cytoplasmic volume is occupied by organelles, chloroplasts, nuclei, and mitochondria, interconnected by a meshwork of fine cytoplasmic strands. Interconnected vacuoles occupy the intervening spaces. The cytoplasm does not stream and cortical and perinuclear microtubules hold the chloroplasts and nuclei in a fixed pattern (Shihira-Ishikawa 1987). The perinuclear microtubule arrays maintain the multiple nuclei at a uniform distance apart (McNaughton and Goff 1990) in an arrangement of optimal packing.

The vacuole of *V. ventricosa* forms a complex interface with the alveolate cytoplasm. Theoretical calculations suggest its membrane surface is “multifolded” by a factor of nine (Wang et al. 1997, Ryser et al. 1999). The vacuole is unusual also in that it contains sulphated polysaccharide mucilages. Sulphated polysaccharides have been identified as one of several wound “plug precursors” in other giant algae (Menzel 1988) and *V. ventricosa* does form “wound plugs” in response to nontraumatic injuries such as a pinprick with a diameter of <100 μm (Nawata et al. 1993). We found that sulphated polysaccharides were intimately involved in the structure of *V. ventricosa* coenocytes (Shepherd et al. 1998, 2001) (Fig. 1c–f).

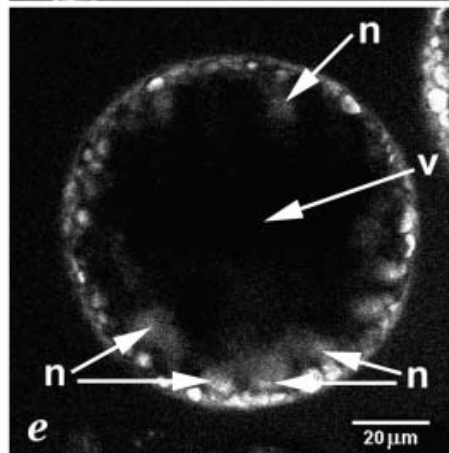
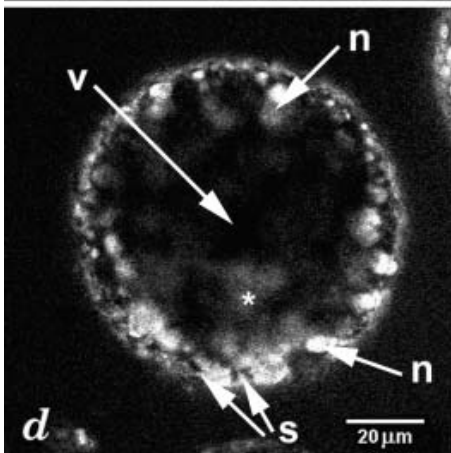
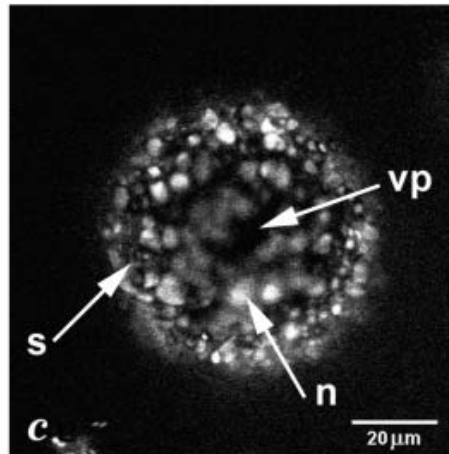
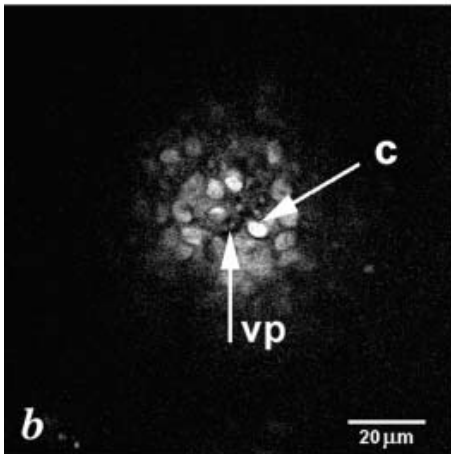
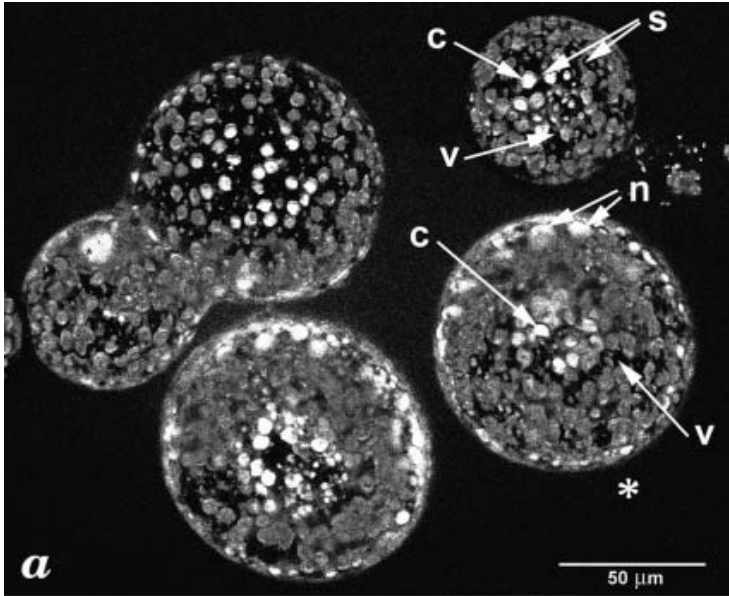
Protoplasts and young cells both contain and are coated in sulphated polysaccharide mucilage, and a communal mucilaginous sheath unites young cells into clusters prior to development of rhizoids (Fig. 1c–e). Plasmolysis of young cells reveals bundles of mucilage-coated filaments spanning the cell wall and connecting intra- to extracellular compartments (Fig. 1f). These filaments resemble the actin-containing cytoplasmic “microvilli” that are remnants of wall-to-membrane linkages in wounded or plasmolysed *Ernodesmis verticillata* cells (Goddard and La Claire 1993). We obtained a three-dimensional representation of the relationship between cytoplasm and vacuole by optically sectioning cells labelled with the fluorochrome 6-carboxyfluorescein (Fig. 2) (Shepherd et al. 2001). We found that the interconnected vacuoles appearing in thin sections for electron microscopy are actually projections from a single, enormously convoluted central vacuole,

Fig. 1a–h. Structure of *V. ventricosa* protoplasts and cells. The process of wound-induced cytoplasmic segregation and protoplast assembly and its time-course are highly conservative. 30 min after cutting a mother cell, inward cytoplasmic contraction and segregation produces swollen cytoplasts connected by fine strands of cytoplasm (about 4 to 7 μm in diameter, spanning distances of up to about 200 μm). The cytoplasts contract into spherical protoplasts after 40 min. Most of the cytoplasm is converted to protoplasts after 1 h. The smallest regenerative protoplasts consistently have a diameter of 10–15 μm . Cell walls form after 3 to 8 h. **a** Contracting cytoplasts (upper arrow) and spherical protoplasts (lower arrow) on a wall segment 40 min after cutting the mother cell. **b** A protoplast (long arrow) formed 40 min after cutting the mother cell. Three elongated chloroplasts (short arrow; compare with the isolated chloroplast, bottom left) within a cytoplasmic strand are entering the protoplast. **c** Sulphated and carboxylated polysaccharides were identified by staining with Alcian Blue at pH 2.5 (Sheehan and Hrapchak 1980). Alcian Blue stains the sulphated polysaccharide mucilage (arrow) coating a protoplast. **d** Sulphated and carboxylated polysaccharides were also identified by staining with 0.2% Toluidine Blue, pH 0.49 (McCully 1970). This 8 h old cell was stained and plasmolysed in glycerol-seawater medium (70:30 [w/v]; osmolarity, 3550 mmol/kg). Toluidine Blue metachromatically stains an extracellular sheath of sulphated polysaccharide mucilage (purple; short arrow). An interior layer orthochromatically stained (blue, long arrow) may be a membrane-cytoskeleton complex. The inset shows mucilaginous contents of the vacuole of a cell gently crushed under a coverslip. Sulphated polysaccharide mucilage is found in the vacuole and extracellular sheath of young walled cells. **e** Toluidine Blue (pH 0.49) stains sulphated polysaccharide mucilage (purple, arrow) in which a cluster of 12 h old walled cells are embedded. The stained mucilage has a fibrous appearance. **f** A 12 h old cell stained with Toluidine Blue at pH 0.49 and plasmolysed. Bundles of stained fine filaments (white arrows, black arrow) span the cell wall (*cw*, arrow) and interconnect the plasmolysed protoplast (*p*, arrows) with the extracellular sulphated polysaccharide sheath (pink). The purple staining of the filaments suggests that they are coated with sulphated polysaccharides. **g** The cytoplasm of young cells was fluorescence-labelled using 6-carboxyfluorescein diacetate, which is membrane permeant and is cleaved by intracellular esterases to the impermeant, highly fluorescent 6-carboxyfluorescein. Fluorescence micrograph (blue exciting light) of a cluster of labelled 3-week-old walled cells. The cytoplasm, including the multiple nuclei (*n*), selectively accumulates the fluorochrome. No autofluorescence of the same wavelengths as 6-carboxyfluorescein was detected in unlabelled cells. **h** Higher magnification of a cell shown in **g**. 6-Carboxyfluorescein is accumulated by the cytoplasm, including chloroplasts (*c*) and nuclei (*n*, overlaid by fluorescing chloroplasts). We interpret the intervening dark (nonfluorescing) regions as protrusions from the central vacuole (*v*) invading the cytoplasm. An extremely thin peripheral layer of cytoplasm probably overlies these regions

which invaginates the alveolate cytoplasm, as do the holes in a sponge.

Ventricaria ventricosa is capable of regulating its turgor pressure (Bisson and Beilby 2002 and references therein). Interestingly the osmotic pressure in the vacuole remains

greater than that in the medium in the early stages of cytoplasmic reticulation and segregation (Nawata et al. 1993) – despite the presence of gaps between retracting cytoplasts. A recent paper (Heidecker et al. 2003) shows that intracellular sulphated polysaccharide mucilage contributes signif-



icantly to resting turgor pressure in *Valonia utricularis*, probably by reducing the chemical activity of water. Gel-associated water with a reduced density becomes K^+ selective (Wiggins and van Ryn 1990).

The unusual electrophysiology of the *V. ventricosa* coenocyte

Aikman and Dainty (1966) speculated that the unusual PD_{vo} of *V. ventricosa* was dominated by a K^+ “pump”. Other researchers reported separate measurements of PD_{vc} and PD_{co} in *V. ventricosa*. Gutknecht (1966) measured a PD_{co} of -71 mV in aplanospores, a PD_{vo} of $+17$ mV in small vacuolate cells, and he estimated PD_{vc} as an unusual $+88$ mV. Davis (1981) directly measured PD_{co} as -70 mV and PD_{vc} as $+86$ mV in small young cells. In contrast, Lainson and Field (1976) found the PD_{co} of aplanospores was only -3.6 to -31 mV. They failed to find negative values for PD_{co} in large mature cells, where PD_{vc} appeared to be zero, but noted that the cell walls of mature cells and aplanospores were significantly negative (-12 mV).

The debate over the electrical properties of *V. ventricosa* appeared to be resolved by Ryser et al. (1999). They measured charge-pulse relaxation spectra of plasmalemma and tonoplast separately, by adding the pore-forming antibiotic nystatin to the outside or to the inside of the cell. The effects of nystatin were transient and variable, and measurements of PD_{co} ranged from -15 to -68 mV, but the results supported Gutknecht (1966) and Davis (1981) rather than Lainson and Field (1976).

Current understanding resolves the small positive PD_{vo} of *V. ventricosa* into a highly unusual PD_{vc} of about $+90$ mV, and a PD_{co} of about -70 mV (Ryser et al. 1999). The PD_{co} is thought to be a K^+ diffusion potential, and the PD_{vc} is attributed to an unusual K^+ pump actively transporting K^+ into the vacuole, as postulated by Hastings and Gutknecht (1974).

However, there are other unusual features of the electrophysiology of *V. ventricosa* that require explanation. The electrophysiological response of the cells to certain stimuli is not only unusual but also inverted when compared to other algal cells. The conductance of *V. ventricosa* cells decreases when the K^+ concentration in the medium is increased (Beilby and Bisson 1999), but the conductance of *C. corallina* cells increases (Beilby 1985). The PD_{vo} becomes more negative in *V. ventricosa* cells experiencing hypotonic conditions and more positive in hypertonic conditions (Bisson and Beilby 2002). These stimuli produce I/V (current as a function of voltage) and G/V (conductance as a function of voltage) characteristics with a marked mirror symmetry (Fig. 3a, b). However, the salt-tolerant charophyte *Lamprothamnium papulosum* experiences PD_{vo} shifts in the opposite direction under hypo- and hypertonic shock, with no obvious symmetry in the I/V and G/V curves (Beilby and Shepherd 2001, Shepherd et al. 2002).

Finally, *V. ventricosa* is not alone in having both unusual electrophysiology and cytoplasmic structure. Other algae of the Cladophorales, *Chaetomorpha darwinii* and *Valoniopsis pachynema*, also have a highly positive PD_{vo} and high tonoplast resistance (Findlay et al. 1971, 1978).

Fig. 2a–e. Cytoplasmic and vacuolar structure in young *V. ventricosa* cells. The figure enables us to demonstrate the wide range of possible locations of microelectrodes inserted to particular depths in cells of different sizes. A cluster of five 18 h old cells was labelled with 6-carboxyfluorescein and optically sectioned at $1.8 \mu\text{m}$ intervals using a Leica DMIRB confocal microscope with Leica TCSNT PC-software. A fluorescein isothiocyanate filter set was used. Unlabelled cells did not autofluoresce in the characteristic yellow-green wavelengths of 6-carboxyfluorescein. The fluorescence of the fluorochrome was only detected in the cytoplasm. **a** Reconstruction of twelve serial optical sections. **b–e** Four optical sections from a representative cell (asterisk in **a**). **a** The reconstruction passes through a range of cell volumes from about 36% (smallest cell, top right-hand side) to about 20% of the diameter of the cell marked by the asterisk. The cytoplasm of all cells has the characteristic alveolate structure. Cytoplasm forms a topologically complex interface with the vacuole. The bulk of the cytoplasm consists of chloroplasts (*c*) and nuclei (*n*) interconnected by fine fluorescing cytoplasmic strands (*s*) that span the dark regions interpreted as projections from the central vacuole (*v*) overlaid by a peripheral cytoplasm only about 40 nm thick (La Claire 1987, Heidecker et al. 2003), in which fluorescence is undetectable. The cytoplasm is a mosaic of thicker and thinner regions. The vacuolar projections (*v*) are continuous with the central vacuole (seen in the smallest cell). **b** Optical section of the most superficial layer of a cell (marked by asterisk in **a**) $0.2 \mu\text{m}$ from its surface. The interconnected chloroplasts (*c*) are partially surrounded by vacuolar protrusions (*vp*) that are continuous with the central vacuole in subsequent sections. The vacuole invaginates the cytoplasm even at this superficial depth (compare with epifluorescence image in Fig. 1h). **c** Optical section $7.4 \mu\text{m}$ from the cell surface. Nuclei (*n*) underlie the chloroplast layer. The vacuolar projection (*vp*) is continuous with that indicated in **b**. Irregular cytoplasmic aggregations associated with nuclei span the vacuole. Thin cytoplasmic strands (*s*) interconnect the cytoplasmic organelles and alternate with vacuolar protrusions extending to the periphery of the image. **d** Optical section $14.6 \mu\text{m}$ into the cell. The central vacuole (*v*) occupies a larger percentage of the volume of the cell and is continuous with the vacuolar projection in **b** and **c**. The cytoplasm in the central region is still spongelike, but it is a sponge with larger holes. Nuclei (*n*) are associated with cytoplasm (asterisk) that penetrates into the centre of the cell. Fine detail of the spongelike cytoplasm–vacuole interface is visible around the edges of the image. **e** Optical section $20 \mu\text{m}$ from the cell surface (20% of the diameter of the cell). The vacuole (*v*) occupies most of the cell interior, continuous with vacuolar protrusions seen in **b–d**. Nuclei (*n*) are still associated with clumps of cytoplasm invaginated by the vacuole. A continuous very fine fluorescent layer of cytoplasm outlines the cell and this may be the thin peripheral cytoplasm

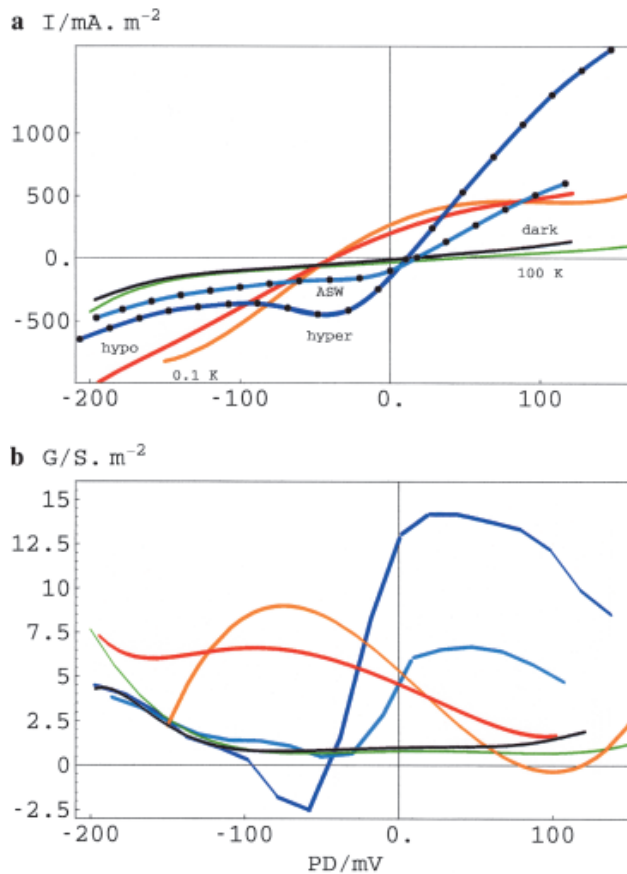


Fig. 3a, b. Electrical characteristics of small (2–3 mm diameter) mature *V. ventricosa* cells under different conditions. The internal PD-measuring electrode was inserted in the cell centre and measured PD_{vo} (the vacuole-to-outside PD). The PD_{vo} was voltage clamped via a Pt-Ir wire terminating near the cell centre, facilitating measurement of the characteristics of current as a function of voltage (I/V) (Beilby 1990). The characteristics of conductance as a function of voltage (G/V) were calculated by differentiation of the I/V profiles (Beilby 1990). **a** I/V profiles; **b** G/V profiles. Typical I/V characteristics in seawater (light blue line) were obtained 2 h after electrode insertion; photosynthetically active radiation of $2.02 \mu\text{mol/s} \cdot \text{m}^2$. The “dark” profile (black line) was obtained after 22 min with a photosynthetically active radiation of $0.5 \mu\text{mol/s} \cdot \text{m}^2$ from the same cell. I/V characteristics following hypertonic shock (increase of osmotic pressure by 100 mosmol/kg for 16 min; dark blue line) and hypotonic shock (decrease of osmotic pressure by 200 mosmol/kg, 28 min exposure; red line) were obtained from different cells. The effects of $[\text{K}^+]_0$ are included for comparison. The green line shows the average profile from 9 cells stabilized in 100 mM K^+ medium, and the orange line shows the average profile from 6 cells stabilized in 0.1 mM K^+ medium (K^+ data replotted from data in Beilby and Bisson 1999: fig. 1). The data can be categorised into three groups: (1) pumping K^+ in (“hyper” and “ASW”, dark and light blue lines) with high conductance at positive values of PD_{vo} ; (2) pumping K^+ out (“hypo”, red line; “0.1 K”, orange line) with high conductance at negative values of PD_{vo} ; (3) low conductance (“dark” and “100 K”, black and green lines) with transporters inactivated or working at low rate. The states 1 and 2 mirror each other. The significance of the similarity between low K^+ and the hypotonic data is yet to be explained

Valoniopsis pachynema also has stationary cytoplasm and highly regular spatial geometry of nuclei established by microtubules (McNaughton and Goff 1990).

A theory relating coenocytic structure to electrophysiological behaviour

Ventricaria ventricosa is a structurally complex organism that stretches to their limits the definitions of “cell”, “cytoplasm”, “vacuole”, “plasmalemma”, and “tonoplast”.

We present a new interpretation of the coenocytic structure *V. ventricosa* (see Fig. 4) on the basis of following arguments.

1. The cytoplasm of *V. ventricosa* is structured from aggregates of uninucleate cytoplasmic domains that can each reconstitute a whole cell. The cytoplasm of the cells consists of interconnected cytoplasmic domains, each of which is a fundamental structural unit, containing a nucleus, chloroplasts, and smaller organelles (e.g., mitochondria, cytoskeleton). Should the organism be damaged or stressed, the cytoplasm is capable of disassociating into the fundamental cytoplasmic domains. Single domains and larger aggregates can regenerate and subsequently reconstitute whole cells. In whole cells the domains are interconnected by a meshwork of fine cytoplasmic strands to form a porous communal cytoplasm interpenetrated by a topologically complex vacuole. The fine dimension of the cytoplasmic strands approaches that of intercellular communication channels.

2. The vacuole and extracellular matrix of young cells contain sulphated polysaccharide mucilage. Both protoplasts and young cells have intra- and extracellular compartments containing acidic sulphated polysaccharide mucilage (Fig. 1c–f). Extra- and intracellular mucilages are likely to make significant contributions to ion and water relations in *V. ventricosa*. In other marine algae, sulphated polysaccharide mucilages reduce hydraulic conductivity, create large “unstirred-layer” effects, and probably have the capacity for ion exchange (Shepherd and Beilby 1999).

3. A communal membrane with inwardly and outwardly directed polarised functions encloses the cytoplasmic domains. The ability of the cytoplasm to segregate, reaggregate, and regenerate new walled cells suggests a communal membrane encloses it. The inward- and outward-facing surfaces of the communal membrane could have polarised K^+ -transporting functions. In this, the cytoplasm would be analogous to a tissue such as the polarised epithelium. The apical part of the communal membrane (plasmalemma) is situated close to the cell wall, appears

smooth (planar) in electron micrographs, and faces the cell wall and mucilaginous matrix, as well as a seawater medium with a high $[\text{Na}^+]/[\text{K}^+]$ ratio. The highly convoluted basolateral part of the membrane (tonoplast) separates the communal cytoplasm from the complex mucilage-containing vacuole with a low $[\text{Na}^+]/[\text{K}^+]$ ratio. The polarity of the communal membrane may be modified in response to environmental factors. There is evidence that the maternal basolateral membrane is transformed into an apical membrane during development of protoplasts, through endocytosis of the maternal apical membrane and exocytosis of membrane material to the basolateral surface (O'Neil and La Claire 1988).

Rationale for arguments

Ventricaria cytoplasm is highly structured into cytoplasmic domains

The extraordinary capacity for wound-induced cytoplasmic regeneration immediately suggests there is something remarkable about the cytoplasm of *V. ventricosa*. Cutting a plant cell open normally results in death, or in the formation of cytoplasmic droplets that are incapable of regeneration, as occurs in *C. corallina*. Yet, although both plasmalemma and tonoplast of a cut *Ventricaria* cell are exposed to seawater of high osmolarity and ionic strength, the cytoplasm responds by contracting into cytoplasts that then regenerate hundreds of walled cells (Fig. 1a). This response is widespread among the members of the Cladophorales, and it is very similar to segregative cell division (La Claire 1982) that occurs in this group of organisms (e.g., in *Dictyosphaeria cavernosa*; Okuda et al. 1997b). The process unfolds in a conservative pattern over a similar time-course in our observations, and in those of others concerning *V. ventricosa* and related algae (Kopac 1933; Doyle 1935; Steward and Martin 1937; La Claire 1982; O'Neil and La Claire 1984, 1988).

This suggests that cytoplasmic segregation and protoplast formation are self-organising processes taking place amongst fundamental units. The smallest protoplast would contain a single unit and larger protoplasts would be made up of multiple units.

What is the nature of the fundamental unit? Only those protoplasts containing at least one nucleus appear to regenerate (Haberlandt 1928, Tatewaki and Nagata 1970). Isolated chloroplasts fail to regenerate. The protoplasts have variable diameters but we find a critical minimum diameter of 10–15 μm . We found respective diameters of 8–10 μm and 5–8 μm for nuclei and chloroplasts, but

Doyle (1935) found some chloroplasts with a diameter of only 2.5 μm . Chloroplasts can also elongate during cytoplasmic segregation (Fig. 1b). Thus, the smallest regenerative protoplast with a diameter of 10–15 μm could contain a single nucleus and as many as six enwrapping chloroplasts, as well as mitochondria and cytoskeletal elements.

We reason that the fundamental regenerative unit is a single cytoplasmic domain containing a nucleus and chloroplasts as well as smaller organelles. Thus the cytoplasmic phase is quantised.

This concept is supported by observations of reproductive differentiation in the related alga *Dictyosphaeria cavernosa*, where the multinucleate cytoplasm is cleaved to form uninucleate zooids through a contractile process that is comparable to wound-induced cytoplasmic contraction (Hori and Enomoto 1978). Multinucleate cytoplasmic aggregates are also partitioned into uninucleate gametes by “protrusions from the vacuole” in *Cladophora flexuosa* (Scott and Bullock 1976).

Studies of the relationship between cytoplasm and nucleus further support the concept of interconnected uninucleate cytoplasmic domains. Nonstreaming coenocytic cells maintain a critical ratio between cytoplasmic and nuclear volumes (McNaughton and Goff 1990, Kapraun and Nguyen 1994). *Ventricaria* cells establish nuclear-cytoplasmic domains of fixed volume (Doyle 1935, McNaughton and Goff 1990).

Vacuole and extracellular matrix contain sulphated polysaccharide mucilage

The cytoplasmic domains within individual cells are embedded in “vacuolar” mucilage, and an extracellular mucilaginous matrix coats the exterior of young cells. Protoplasts both contain and are coated in sulphated polysaccharide mucilage. Wall-spanning filaments interconnect intra- and extracellular compartments, and the cells are clustered and embedded in a communal mucilage (Fig. 1e, f).

Similarly to *V. ventricosa*, wound-induced protoplast formation takes 1–1.5 h in *B. forbesii* and the primary cell wall is deposited only 2–3 h after wounding (Itoh et al. 1984). The outer protoplast membrane quickly becomes a functional plasmalemma capable of synthesising a cell wall. Extracellular sulphated polysaccharide mucilage is present both before and after wall synthesis in *V. ventricosa*. Persistent linkages between the plasmalemma and cell wall tether the contracting cytoplasm to the cell wall in both wounded *Ernodesmis verticillata* (Goddard and La Claire 1993) and plasmolysed *Ventricaria* cells (Fig. 1f). The membrane-bound cytoplasm is everywhere

found in close proximity to sulphated polysaccharide mucilage, even in these fine extensions.

What is the significance of this mucilage? We have previously shown that such mucilage significantly impacts upon the cellular response to osmotic shock. Sulphated polysaccharide mucilage provides a negatively charged apoplastic barrier that reduces or prevents the opening of Ca^{2+} and Ca^{2+} -activated Cl^- ion channels in response to hypotonic shock (Shepherd and Beilby 1999). Mucilage provides a significant unstirred layer and reduces hydraulic conductivity (Shepherd et al. 1999). Mucilaginous cells respond to hypotonic shock primarily through mechanosensitive ion channels (Shepherd et al. 2002). Sulphated polysaccharide mucilage in the vacuole of *Valonia utricularis* significantly contributes to steady-state turgor pressure, probably by reducing the activity of water (Heidecker et al. 2003). The mucilage could also contribute to the exclusion of Na^+ and accumulation of K^+ by the vacuole observed by Shihira-Ishikawa and Nawata (1992). An interesting series of experiments (Wiggins and van Ryn 1990, Wiggins 1995) shows that gel-associated water has a lower density than external water, and this gel-water becomes K^+ selective. Thus, the mucilage has a potent effect on ion and water relations that was not considered by early researchers.

The vacuole fails to accumulate 6-carboxyfluorescein from the cytoplasm (Figs. 1g, h and 2); although, the vacuoles of most higher-plant cells (Wright and Oparka 1994), charophyte internodal cells (Shepherd and Goodwin 1992, Beilby et al. 1999), and fungal hyphae (Shepherd et al. 1993) rapidly accumulate this fluorochrome. Vacuolar accumulation of xenobiotics in plants and fungi has recently been attributed to tonoplast-specific ABC transporters (Rea 1999). The lack of characteristic vacuolar transporter systems, the accumulation of mucilage, the presence of a highly unusual K^+ pump (Hastings and Gutknecht 1974), and the amplified surface area of the membrane (Wang et al. 1997, Ryser et al. 1999) all make sense if the inner membrane of *V. ventricosa* is indeed not a true tonoplast. We will argue that it is the inner face of a polarised communal membrane.

Communal membrane with inwardly and outwardly directed polarised functions encloses cytoplasmic domains

The remarkable capacity for cytoplasmic segregation and reassembly into regenerative protoplasts is difficult to understand if, as for *C. corallina*, the tonoplast and plasma membrane are viewed as essentially separate membranes.

The preservation of cytoplasmic integrity after cutting could be explained if the membranes are not actually severed, but deformed, or if they reseal immediately after cutting (Menzel 1988). However, the fact that protoplasts develop cell walls within 2–3 h (Itoh et al. 1984) is difficult to explain if a separate plasmalemma and tonoplast initially enclose the maternal cytoplasm. The inner membrane (tonoplast) would have to fuse with the outer membrane (plasmalemma) to form a single plasmalemma capable of synthesising a cell wall.

How is the maternal tonoplast transformed into the protoplast plasmalemma? Our argument is twofold. (1) The polarity of function of apical and basal faces of the communal membrane is stabilised by the microtubule arrays in whole cells. Environmental factors including wounding, and hyper- or hypoosmotic stress disrupt the microtubule arrays, thus disrupting the polarity between apical and basolateral membranes. (2) The apical surface could contract and the basolateral surface expand, through processes of membrane recycling involving endocytosis and exocytosis that have been elegantly demonstrated by O'Neil and La Claire (1988).

Stabilisation of the polarity of the communal membrane by cytoskeletal arrays

It is clear from the literature that the microtubule cytoskeleton is involved in maintaining the cytoplasmic structure in whole cells. The behaviour of cortical and perinuclear microtubule arrays is very different from that of microtubule arrays of higher-plant cells. The perinuclear arrays are persistent (La Claire 1987), while they are present only prior to mitosis in higher-plant cells (Wasteneys 2002). Unlike those of higher-plant cells, the cortical arrays are spatially separate from the perinuclear arrays and are unchanged throughout the nuclear cycle (La Claire 1987).

The unusual distribution and persistence of microtubule arrays suggests a role both in maintaining spatial relationships between cytoplasmic domains and in spatially organising cytoplasmic segregation into regenerative protoplasts. Figure 4a and b shows the arrangement of perinuclear and cortical microtubules, in relation to the array of nuclear centres, to the chloroplasts closer to the surface, and to the proposed cytoplasmic domains. The structure of a single cytoplasmic domain is shown in Fig. 4c.

We propose that perinuclear microtubule arrays connect the domains in whole cells (Fig. 4a), maintain the structural integrity of individual domains (Fig. 4c), and thereby maintain the polarity of the communal membrane sur-

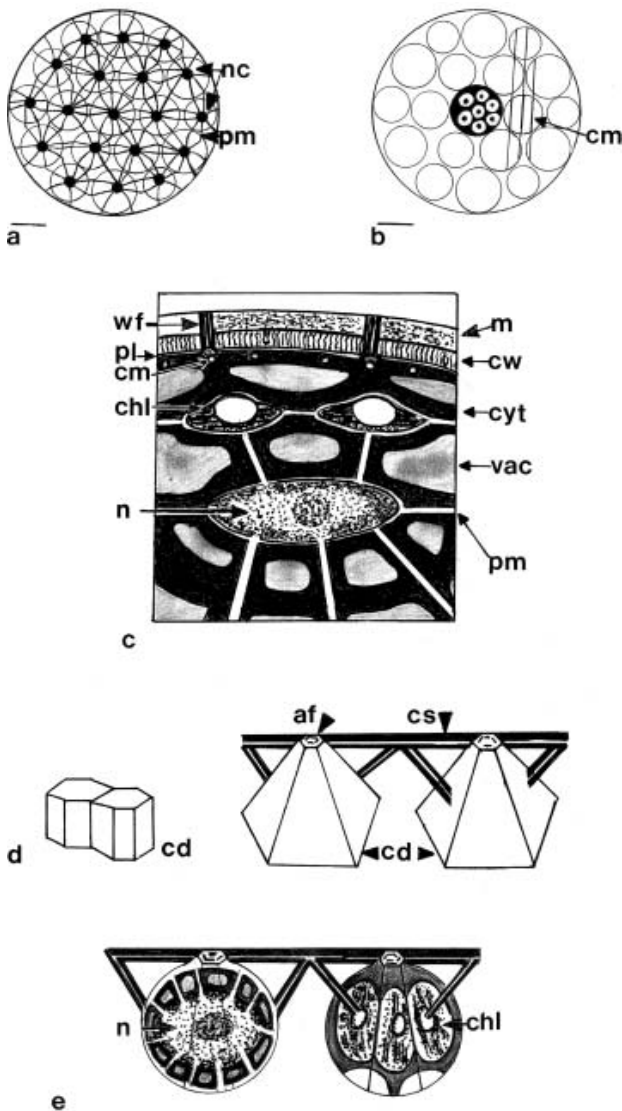


Fig. 4a–e. Diagrammatic and schematic representations of the hypothesised structure of cytoplasmic domains in *V. ventricosa* cytoplasm. **a** Outline of a cell traced from Fig. 2 to show relationships between perinuclear microtubules (*pm*), nuclear centres (*nc*), and the postulated cytoplasmic domains. The nuclear centres are maximally spaced (McNaughton and Goff 1990). The cytoplasmic domains are represented as circles enclosing the nuclear centres. The actual geometry of the domains is not known, although they maintain a constant ratio between nuclear and cytoplasmic volumes. Most nuclei have either five or six neighbours in whole cells. The nuclei lie beneath the chloroplasts and are interconnected by cytoplasm-coated perinuclear microtubules that span the domain boundaries. Approximate scale bar, on the basis of Fig. 2, is 20 μm . The radiating pattern of perinuclear microtubules is based on micrographs of La Claire (1987) and Shihira-Ishikawa and Nawata (1992). **b** Outline of the same cell showing relationships between cortical microtubules (*cm*), chloroplasts (white circles in the central cytoplasmic domain), and the postulated cytoplasmic domains. Chloroplasts are situated closest to the cell wall and are surrounded by either five or six neighbours. The parallel cortical microtubules that occupy the thin surface layer of cytoplasm are depicted as straight lines. The pattern of cortical microtubules is based on fluorescence micrographs of La Claire (1987) and Shihira-Ishikawa and Nawata (1992).

rounding the cytoplasmic domains. Perinuclear microtubules are greatly shortened 30 min after wounding (Shihira-Ishikawa and Nawata 1992) when protoplasts are developing. This would facilitate the separation of the fundamental cytoplasmic domains so that individual protoplasts containing at least one nucleus could develop. The polarity of the communal membrane would temporarily disappear as protoplasts develop.

Maternal cortical microtubules persist in cytoplasmic strands connecting developing protoplasts (La Claire 1987). They could maintain the orientation of the chloroplasts. Depolymerisation of cortical microtubules leads

Long cortical microtubules are regularly spaced (2 to 4 μm apart) in whole *B. forbesii* cells (La Claire 1987). This may reflect regular spacing of the chloroplasts in the theorised domains. **c** Diagrammatic view of a transverse section of a single cytoplasmic domain. Extracellular sulphated polysaccharide mucilage (*m*) coats the cell wall (*cw*). Wall-spanning filaments (*wf*) connect the protoplast to the mucilage; these are shown in close proximity to cortical microtubules (*cm*) that occupy the extremely thin layer of cytoplasm enclosed by plasmalemma (*pl*) or the apical portion of the cell membrane. The tonoplast or basolateral portion of the membrane faces the vacuolar compartment (*vac*). The vacuole invaginates the thin cytoplasmic layer (*cyt*) that coats the organelles (chloroplasts [*chl*] and nucleus [*n*]). The nuclei are interconnected by cytoplasm-coated perinuclear microtubules (*pm*) that span the boundaries of the cytoplasmic domains. The tonoplast or basolateral membrane has a greater surface area than the plasmalemma or apical membrane. The spongelike structure of the cytoplasm–vacuole interface is based on the electron micrographs and diagram by Shihira-Ishikawa and Nawata (1992). **d** Hypothesised mechanism by which cytoplasmic contraction produces spherical protoplasts. The cytoplasmic domains (*cd*) are represented diagrammatically as interconnected solids. Following wounding, actin filaments (*af*) in the peripheral cytoplasmic layer contract the apical but not the basal surfaces of the cytoplasmic domains. This forms a reticulum of actin filaments (seen in La Claire 1989) when viewed from above. At the same time, the apical-membrane material is endocytosed and exocytosed to the basolateral membrane (as described by O’Neil and La Claire 1988). Cortical microtubules persist in cytoplasmic strands (*cs*) that connect the cytoplasmic domains. Perinuclear microtubules are shortened, disconnecting the cytoplasmic domains from one another and enabling formation of cytoplasts and protoplasts. The process works through contraction of the upper surface of cytoplasm and endocytosis of the apical membrane (plasmalemma), without contraction of the basolateral regions of cytoplasm, but with exocytosis of membrane material to the basal membrane. This would result in the formation of spherical protoplasts enclosed by a membrane that is essentially a maternal “tonoplast” transformed by endocytosis and exocytosis into a functional “plasmalemma”. O’Neil and La Claire (1988) showed that the new “tonoplast” is probably constructed from recycled maternal “plasmalemma”. **e** Diagrammatic representation of developing protoplasts. For simplicity, single cytoplasmic domains are shown contracting into the smallest protoplasts observed (about 10–15 μm diameter). The sphere on the left-hand side shows the interior of such a protoplast. The nucleus (*n*) retains short cytoplasm-coated perinuclear microtubules and mucilage invaginates the cytoplasm as in the postulated domains of whole cells (drawn and shaded as in **c**). The sphere on the right-hand side shows the surface view, where there is space for six flattened chloroplasts (*chl*). The thin cytoplasmic strands contain cortical microtubules. These maintain the parietal orientation of chloroplasts

to disordering and clumping of chloroplasts (La Claire 1987). A radial array of cortical microtubules develops as the first sign of lenticular cell formation in *Valonia utricularis* and provides positional information for cytoplasmic aggregation, including chloroplasts, at a specific site (Okuda et al. 1997a). The wall–membrane tethers found in contracting *Ernodesmis verticillata* cytoplasm contained actin microfilaments (Goddard and La Claire 1993) and similar wall-spanning filaments in plasmolysed *V. ventricosa* (Fig. 1f) could anchor portions of the contracting apical surface to the cell wall.

Membrane recycling and the reversal of polarity of the communal membrane

The peripheral cytoplasm in *V. ventricosa* and related algae is extremely thin (about 40 nm thick) and it is separated from the cell wall by a smooth, planar membrane (Hori and Enomoto 1978, La Claire 1987, Shihira-Ishikawa and Nawata 1992), the apical portion of the communal membrane (Fig. 4b). The thin peripheral cytoplasm contains the cortical microtubules (Shihira-Ishikawa and Nawata 1992) that persist during cytoplasmic segregation (La Claire 1987).

The highly convoluted membrane lining the inner face of the alveolate cytoplasm is the basolateral membrane (Fig. 4c). The basolateral membrane separates the peripheral and internal cytoplasm from the vacuole and coats the cytoplasm containing the perinuclear microtubule arrays that stabilise the cytoplasmic domains.

Cytoplasmic contraction is facilitated by the actin cytoskeleton (La Claire 1989). The process does not depend on microtubules (La Claire 1987). The actin cytoskeleton is transformed from a punctate pattern to a wide-meshed reticulum of bundles about 45 min after wounding, as protoplasts develop (La Claire 1989). Fine cytoplasmic strands (La Claire 1987: fig. 12) containing dozens of maternal cortical microtubules as well as organelles span the gaps between regions of contracting cytoplasm in *B. forbesii*. We have observed identical strands connecting regions of contracting *Ventricaria* cytoplasm 45 min after cutting the mother cell (not shown). The actin cytoskeleton is associated with movement of cytoplasm and organelles, whilst the cortical microtubules provide positional information. Cortical microtubules control the position but not the movement of chloroplasts to a specific site during lenticular cell formation in *Valonia utricularis* (Okuda et al. 1997a).

We propose that the actin filaments contract the peripheral (apical) but not the internal (basolateral) regions of the cytoplasmic domains (Fig. 4d, e), at the same time as the perinuclear microtubules shorten and domains sepa-

rate. This would result in inwardly facing cytoplasmic protrusions connected by cytoplasmic strands that contain maternal cortical microtubules. The basolateral regions of the cytoplasmic domains, not being contracted by actin bundles, would correspond to the inwardly swollen nascent protoplasts. The nascent protoplast would then be enclosed largely by the maternal tonoplast (basolateral membrane; Fig. 4d, e). If this is to occur, the surface area of the maternal plasmalemma and tonoplast must change drastically: the former decreasing, the latter increasing.

A fascinating series of papers already shows that this can occur. First, O'Neil and La Claire (1984) showed that numerous coated pits and vesicles form in proximity to the plasmalemma during wound-induced cytoplasmic contraction in *B. forbesii*. These organelles are associated with the endocytosis of large amounts of the plasmalemma. In a further analysis, O'Neil and La Claire (1988) used cationic ferritin as a marker of surface membranes and analysed membrane dynamics during cytoplasmic contraction and protoplast formation in *B. forbesii*. They produced powerful evidence for membrane recycling. As protoplasts developed, endocytosis of the maternal plasmalemma was coupled with exocytosis and insertion of membrane material in the maternal tonoplast. After 90 min, the spherical protoplasts had become distinct entities evenly coated with ferritin and the former tonoplast and plasmalemma were indistinguishable. At this point ferritin-containing cytoplasmic vesicles began to fuse to form a new central vacuole. The time-course of ferritin incorporation (see O'Neil and La Claire 1988: fig. 5) suggests that the new tonoplast might form from recycled maternal plasmalemma. The new surface membrane, a blend of maternal tonoplast and recycled plasmalemma, then functions as a plasmalemma and a cell wall forms soon after, at 120 min (O'Neil and La Claire 1988).

The actin-induced contraction of the superficial cytoplasm is thus tightly coupled with endocytosis of the maternal plasmalemma material and the exocytosis of membrane material to the former tonoplast, which encloses the nascent protoplasts. Plasmalemma can be recycled and merged with the tonoplast to form a new plasmalemma, and it can be recycled to form a new tonoplast. This supports our concept of a communal membrane whose basolateral and apical surfaces enclose the interconnected cytoplasmic domains in whole cells.

Although the cytoskeletal arrays behave differently from those in higher plants, they have some similarities to a tissue such as the polarised epithelium. Polarised epithelia (Alberts et al. 1998) also have a system of actin bundles at the apical end of the cell, a system of microtubules

running parallel to the cell axis (like the perinuclear microtubules connecting chloroplasts to nuclei) and a system of microtubules running parallel to the apex of the cell (like the cortical microtubules). This cytoskeletal arrangement facilitates the process of epithelial invagination, which resembles that of protoplast formation.

Nature of the interconnections between cytoplasmic domains

Electron micrographs (La Claire 1987: fig. 2, Heidecker et al. 2003) show that cortical microtubules are located in a peripheral cytoplasmic layer only about 40 nm thick. Since microtubules have outer and inner diameters of about 25 nm and about 14 nm, respectively, space permits only lateral arrangements of single cortical microtubules in the peripheral cytoplasm, with only about 15 nm of cytoplasm interposed between microtubule surface and membrane. The peripheral cytoplasm is so thin that its width approaches the gross diameter of simple plasmodesmata (see Overall and Blackman 1996: fig. 1). The finest strands of the cytoplasmic meshwork interconnecting cytoplasmic domains (estimated from Shihira-Ishikawa and Nawata 1992: figs. 2 and 3) are 80 to 100 nm in diameter, and these strands also contain perinuclear microtubules. The strands have a diameter similar to that of the pores of the intercellular communication channels in a basidiomycete fungus (about 70 nm; Shepherd et al. 1993). As an indicator of how fine these interconnecting strands actually are, the diameter of the nuclear-pore complex is 83.9 nm (Jaggi et al. 2003). Communication between cytoplasmic domains through the fine interconnecting cytoplasmic strands might have some properties in common with communication through plasmodesmata, nuclear pores, and other intercellular channels. Communication between domains is potentially “gated” and could have a molecular-size exclusion limit.

Meaning of electrophysiological data obtained from a coenocyte

How can the structural analysis described above be reconciled with the electrophysiology of *V. ventricosa*? The very concept of PD_{co} relies on the cytoplasm being a relatively homogeneous phase, which is clearly not the case for *V. ventricosa* (Fig. 2). The lateral conductivity of the interconnections between the cytoplasmic domains has not been studied, but if it is low, or variable, PD_{co} will not have a single unique value. Furthermore, the membrane trafficking (O’Neil and LaClaire 1988) blurs the distinction between the outer and the inner membranes. The

mosaic of thicker and thinner regions of cytoplasm interpenetrated by the convoluted vacuole (Fig. 2) probably explains the wide range of PD measurements obtained by different researchers. It is highly unlikely that a microelectrode with tip diameter of about $1\ \mu\text{m}$ will impale the 40 nm thick peripheral cytoplasm or the about 80 to 300 nm diameter interconnecting cytoplasmic strands. Such a microelectrode will miss the fine cytoplasmic meshwork and peripheral cytoplasm and enter nuclei and/or chloroplasts (Fig. 2). The negative values for PD_{co} found by Davis (1981) and Gutknecht (1966) could be the PD of cytoplasmic organelles. The transience of negative PDs measured by Ryser et al. (1999) could be due to repair of the nystatin-treated membrane through membrane endocytosis and exocytosis (O’Neil and La Claire 1988).

However, if the microelectrode is inserted into the centre of the cell, it is reasonably certain that it crosses the complex cytoplasm into a vacuolar region (Fig. 2). The centre of the smallest cell in Fig. 2a is occupied by vacuolar material. This microelectrode placement would give PD_{vo} measurements that are akin to a tissue potential and integrate over all of the cytoplasmic phases and domains. Using a central microelectrode placement, we found that small cells gave consistent vacuole-to-outside I/V behaviour, in seawater and under a range of different conditions (Fig. 3) (Beilby and Bisson 1999, Bisson and Beilby 2002).

The cells are thought to regulate turgor pressure through ATP and pressure-dependent K^+ and Cl^- transporters in the plasmalemma (Heidecker et al. 2003). The vacuole accumulates K^+ and Cl^- (K^+_{vac} and Cl^-_{vac} are respectively 21 times and 1.2 times their concentrations in seawater) but excludes Na^+ (Na^+_{vac} is only about 15.6% of the seawater concentration; Shihira-Ishikawa and Nawata 1992). The putative K^+ pump is expected to maintain this concentration imbalance in seawater. The I/V and G/V curves (Fig. 3a, b; pale blue line) show that a cell in seawater is more conductive at positive PD_{vo} . In hypertonic medium (Fig. 3a, b; dark blue line) the K^+ pump is expected to work harder to increase the internal K^+ concentration, and the cell conductance does indeed increase, with a positive PD_{vo} generated by the import of K^+ . This is analogous to the negative PD_{vo} in charophytes and higher-plant cells resulting from export of H^+ by the proton pump. On the other hand, we expect K^+ to be exported in hypotonic medium, and, as expected, the conductance increases, with a negative PD_{vo} generated by K^+ export (Fig. 3a, b; red line). These results could potentially be explained by a reversal of the polarity of the K^+ pump in situ or by its migration to the other membrane surface through endo- or exocytosis.

In the dark (Fig. 3a, b; black line) the cell has a PD_{vo} close to zero and low conductance at the resting PD. Similar I/V profiles are obtained when the cell is exposed to metabolic inhibitors that result in depletion of ATP (M. J. Beilby and M. A. Bisson unpubl.) suggesting that the polarised ion transport is ATP dependent. When exposed to a medium with high K^+ concentration, the cell conductance declines, but PD_{vo} remains positive at about +50 mV (Fig. 3a, b; green line). Conversely, the PD_{vo} becomes negative and the conductance slightly increases in a medium with low K^+ concentration (Fig. 3a, b; orange line). We initially interpreted the response to changes of $[K^+]_o$ in terms of a K^+ channel-dominated plasmalemma, with a large positive offset due to a K^+ pump on the tonoplast membrane (Beilby and Bisson 1999). However, the E_K was calculated using the cytoplasmic K^+ concentration of 434 mM (Gutknecht 1966), which is likely to be an overestimate due to the complex cytoplasmic structure. These results could also be interpreted in terms of a reversal of polarity between inner and outer membrane and/or movement of the K^+ pump between basolateral and apical membranes through endo- or exocytosis.

The I/V and G/V curves in seawater, low K^+ , and hypotonic and hypertonic conditions show a marked mirror symmetry across the PD spectrum. The hyper- and hypotonic curves show a mirror symmetry in comparison with each other. These are features we might expect of a communal membrane that is dominated by polar K^+ transport and which can reverse its polarity or even interchange its surfaces following environmental stress.

Conclusion

There are key features of the *V. ventricosa* coenocytic structure that are essential for understanding its electrophysiology. First, the cytoplasm is structured into uninucleate domains that can separate to regenerate new cells and which behave collectively as a tissue. Second, the cytoplasmic domains are bounded by a communal membrane with basolateral and apical faces whose functions are polarised in whole cells. Third, the basolateral and apical membranes can recycle components and transform into one another in response to stress. Finally, interior and exterior mucilages profoundly influence ion and water transport.

At first glance the ability of *V. ventricosa* to form new cells from fragments of cytoplasm seems to contradict Virchow's dictum that all cells must come from a preexisting cell. However, a coenocyte can be viewed as a gestalt. According to Esau (1965), a coenocyte is an aggregation of units, each consisting of a nucleus and adjacent cyto-

plasm and each representing a cell. Electrophysiological measurements of PD_{co} in such a coenocyte are unreliable, but measurements of PD_{vo} constitute a tissue potential that reflects polarised transcytoplasmic K^+ transport from apical to basolateral membranes. This interpretation will open new directions for electrophysiological research.

Note added in proof. The "spongy" structure of the *Valonia utricularis* cytoplasm was also described in a recent paper by S. Mimietz et al. (*Protoplasma* 222: 117–128, 2003).

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