Eur Biophys J (2002) 31: 341–355
DOI 10.1007/s00249-002-0222-6

ARTICLE

V.A. Shepherd · M.J. Beilby · T. Shimmen

Mechanosensory ion channels in charophyte cells: the response to touch and salinity stress

Received: 21 January 2002 / Revised: 1 March 2002 / Accepted: 8 March 2002 / Published online: 13 June 2002

Abstract Mechanosensitive (MS) ion channels are activated by mechanical stress and then transduce this information into electrical signals. These channels are involved in the growth, development and response to environmental stress in higher plants. Detailed analyses of the electrophysiology in higher plants are difficult because such plants are composed of complex tissues. The large cells of the charophytes facilitate electrophysiological measurements and allow us to study MS ion channels at the level of single cells. We draw parallels between the process of touch-perception in freshwater *Chara*, and the turgor-regulating response to osmotic shock in salt-tolerant *Lamprothamnium*. In terms of electrophysiology, these responses can be considered in three stages: (1) stimulus perception, (2) signal transmission and (3) induction of response. In *Chara* the first stage is due to the receptor potential (RPD), a transient depolarization with a critical threshold that triggers action potentials, which are responsible for stages (2) and (3). Receptor potentials are generated by MS ion channels. Action potentials involve a transient influx of Ca^{2+} to the cytoplasm, effluxes of K^+ and Cl^- and a temporary decrease of turgor pressure. Reducing cell turgor increases sensitivity to mechanical stimulation. In *Lamprothamnium*, a hypotonic shock produces an extended depolarization that resembles an extended RPD and is responsive to osmotic rather than ionic changes. Like the action potential, a critical threshold depolarization triggers Ca^{2+} influx, opening of Ca^{2+}-sensitive Cl^- channels and K^+ channels; effluxes that last over an hour and result in turgor regulation. These processes show us, in primal form and at the level of single cells, how mechanoperception occurs in higher plants. Recent progress in research into the role of MS ion channels in the freshwater and salt-tolerant Characeae is reviewed and the relevance of these findings to plants in general is considered.

Keywords Mechanosensory ion channel · Charophytes · Turgor regulation · Plant action potential · Plant receptor potential

Presented at the Australian Biophysical Society Meeting, 2001

V.A. Shepherd (✉) · M.J. Beilby
UNESCO Centre for Membrane Science and Technology,
School of Physics, Biophysics,
The University of New South Wales,
Sydney, NSW 2052, Australia
E-mail: vas@newt.phys.unsw.edu.au
Fax: +61-2-93854484

T. Shimmen
Department of Life Sciences,
Faculty of Science, Himeji Institute of Technology,
Harima Science Park City, Hyogo 678-12, Japan

Introduction

The response to touch, injury and other forms of mechanical stimulus in plants has been researched since the 1900s (reviews: Pickard 1973; Hill and Findlay 1981; Wayne 1994; Shepherd 1999; Shimmen 2001a). Perception of mechanical stimuli occurs at the level of mechanosensory ion channels (MS channels) that are activated by mechanical stress on the cell membrane and cell wall. The molecular structure and function of MS channels has been intensely researched in animal cells and bacteria (Hamill and Martinac 2001) and we will focus on the function of MS channels at the level of the electrophysiological behaviour of whole plant-like cells. MS channels transduce mechanical information into electrical signals, such as receptor potentials (RPD) and subsequently action potentials, in giant charophyte algae (Shimmen 1996). MS channels are widespread and play a role in touch perception, turgor regulation and/or cell volume regulation in cells as diverse as bacteria (Chamberlin and Strange 1989), the bovine lens cell (Srinivas et al. 1998), the salt-tolerant charophyte *Lamprothamnium* (Shepherd and Beilby 1999) and the guard cells that control the stomatal aperture, and hence gas exchange and transpiration in higher plants (Cosgrove and Hedrich 1991). On the whole, these

disparate cells use K^+ as the main inorganic osmoticum, and K^+ is the main cation expelled or taken up during turgor and/or volume regulation, accompanied by Cl^- or other anions. The ability to respond to touch or changes in osmotic pressure clearly originated very early during cellular evolution and the MS channels involved are probably highly conserved.

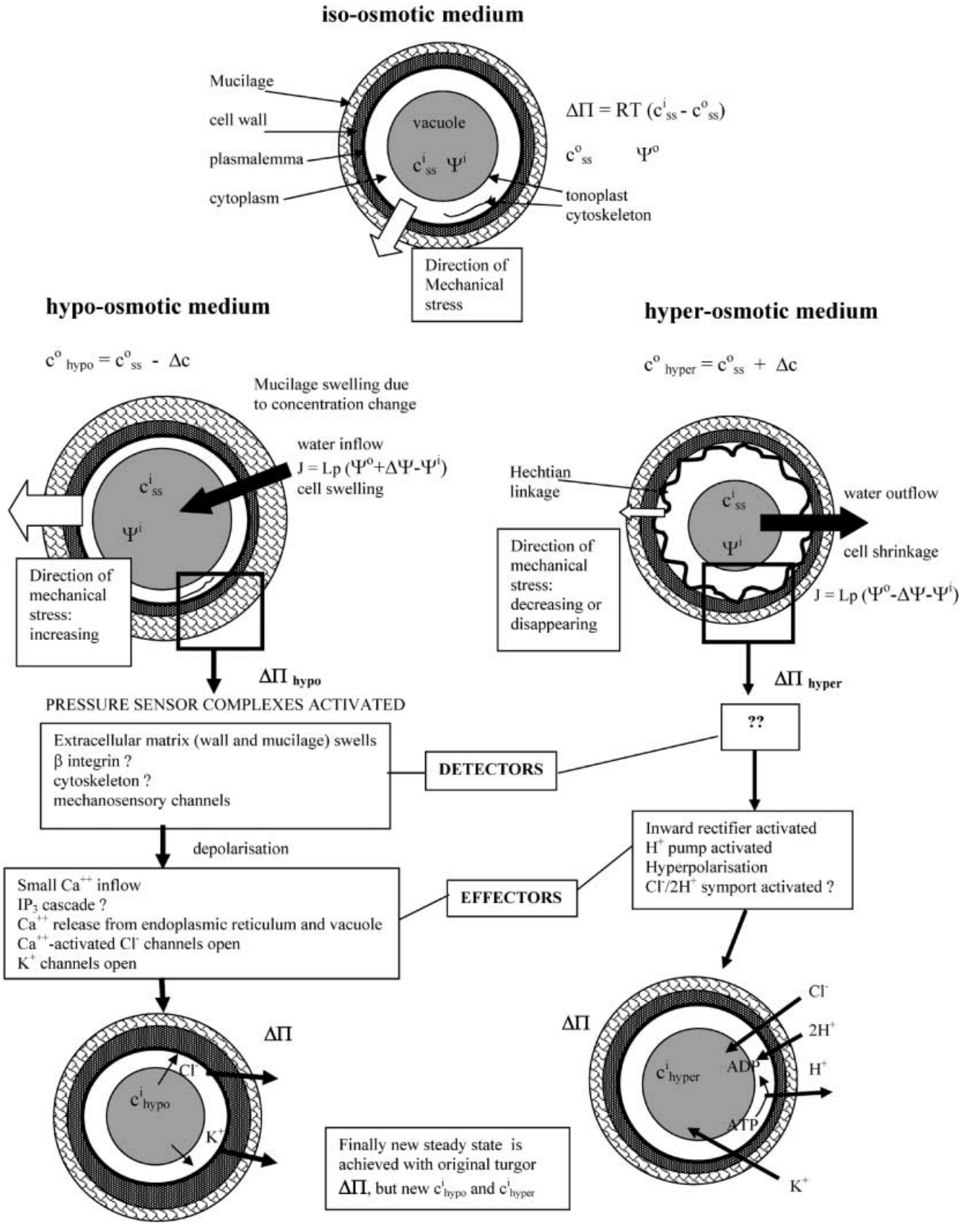
The importance of turgor regulation and the need for mechanoreceptors in higher plants

Plants have elaborated this ancient cellular capacity for controlling the balances of K^+ and associated anions, often Cl^-, into behavioural repertoires involving movements of cells and organs (see review by Findlay 2001). Plant cells are enclosed in cell walls, and form rigid collective structures that are unified by turgor pressure. The cell walls of the plant body form an extracellular matrix, a continuum called the apoplast, a wet cellulosic space that can conduct action potentials and is impermeable to water only at casparian strips and suberized lamellae. The apoplast encloses the symplast, the continuum of protoplasts that are interconnected by plasmodesmata, wall-spanning intercellular communication channels. Turgor pressure is the pressure exerted outwardly on the relatively inelastic cell wall by the protoplast. Water flow between apoplast and symplast across the cell membrane is described by $J = Lp(\Psi^i - \Psi^o)$, where Lp is the hydraulic conductivity of the wall/membrane complex, and Ψ^i and Ψ^o are the water potentials inside and outside the cells. The difference in water potentials (Ψ^i–Ψ^o) equates to the difference between hydrostatic and osmotic pressures inside and outside the cell ($\Delta P = P^i - P^o$)–($\Delta\Pi = \Pi^i - \Pi^o$), as shown in the schematic diagram in Fig. 1. In the homeostatic situation, where there is no net water flux across the cell membrane, the hydrostatic pressure difference is equal to the osmotic pressure difference, and the turgor pressure $\Delta\Pi$ is then equal to the pressure generated by the difference between intracellular (symplastic) and extracellular (apoplastic) osmotic pressures. The major determinants of turgor pressure then are Y, the Young's modulus for elasticity of the cell wall, and Π^i–Π^o, the difference in osmotic pressure in the symplast and the apoplast. Any form of controlled movement in plants, either by growth, response to injury, or nastic movements, implies changes in and regulation of turgor pressure.

During turgor regulation the major ion fluxes occur between vacuole and apoplast, with the cytoplasm being a route for rapid transit (see Fig. 1). As a physical property the turgor pressure is propagated through the whole plant body, and at the whole plant level it is regulated by symplastic and apoplastic barriers. If adjoining cells have different turgor pressure then the plasmodesmata between them are disconnected (Ding and Tazawa 1989), interrupting the symplast. This may occur when groups of cells begin to pursue a different differentiation pathway. For example, plasmodesmata connecting developing reproductive structures are plugged prior to spermiogenesis in *Chara* (Kwiatkowska 1988; Shepherd and Goodwin 1992). On the other hand, casparian strips and suberized lamellae force water and solutes into a symplastic path at critical entry points (roots) or exits (leaves) of higher plants (Canny 1990). Living cells provide a compensating pressure that forces water into embolized xylem vessels in a recently proposed compensating pressure theory (Canny 1995, 1998). Living cells of rays or xylem parenchyma were predicted to have the capacity for pressure sensing, through stretch receptors (Canny 1995) or MS channels. Not only does turgor pressure regulation control the "skeletal" system of the plant, and transpiration and gas exchange through stomata, it is itself feedback regulated according to growth and development needs of different parts of the plant body. Any cell throughout the plant which is exposed to varying solute concentrations c^o needs to be able to respond to changes of $\Delta\Pi$.

The apoplast serves as a reservoir for ions and water during plant cell or organ movements that involve changes in turgor pressure. In land plants the actual ion concentrations "seen" by the plasmalemma depend on complex surface charge properties of the apoplast (Kinraide 2001), whilst it is usually assumed that cells of aquatic plants have access to an unlimited apoplast. This assumption applies to freshwater algae living in dilute environments but is certainly not true in marine algae, with complex elaborations of the cell wall (Shepherd and Beilby 1999). Turgor pressure is regulated by controlling the movements of predominantly K^+, accompanied by Cl^- or other anions, between symplast and apoplast. In plant cells the majority of the K^+ and Cl^- of the symplast is stored in the vacuole, which, in the resting state, normally contains high concentrations of K^+, Cl^- and Ca^{2+}. Water-filled intercellular spaces contain

Fig. 1. Schematic diagram of turgor regulation. In a steady state the turgor pressure, $\Delta\Pi$, arises from the differences between external and internal solute concentrations, c^i_{ss} and c^o_{ss}, respectively. The limited elasticity of the cell wall prevents water flow, and the water potentials inside and outside, Ψ^i and Ψ^o, respectively, are balanced by the hydrostatic pressure difference ΔP ($= P^i - P^o$). Under most conditions this pressure is directed outwards. With no water flow, $\Delta P = \Delta\Pi = RT(c^i_{ss} - c^o_{ss})$, where R is the universal gas constant and T is the temperature in K. Cells in different plants and parts of plants exhibit a range of steady-state turgor pressures. During hypotonic challenge the outside concentration of solutes decreases by Δc, the water potentials no longer balance, and water flows into the cell: $J = Lp(\Psi^i + \Delta\Psi - \Psi^o)$, where Lp is the hydraulic conductivity. This flow stops within seconds, once again limited by the cell wall (c^i unchanged to a first approximation), but a greater hydrostatic pressure difference (and greater $\Delta\Pi_{hypo}$) results, once again balancing the water potentials. However, this situation might be dangerous for the cell, as the wall might burst. Many cells can detect the increase in $\Delta\Pi$, opening channels and decreasing c^i until $\Delta\Pi$ returns to some comfortable value. The details of this regulation are discussed in the text. During hypertonic challenge the water flows out of the cell, decreasing or totally abolishing $\Delta\Pi$. In this case the cell has to expend metabolic energy to increase c^i until normal $\Delta\Pi$ is re-established. The details of the process are discussed in the text


iso-osmotic medium
Mucilage
cell wall
plasmalemma
cytoplasm
vacuole
c^i_{ss} Ψ^i
$\Delta\Pi = RT\ (c^i_{ss} - c^o_{ss})$
c^o_{ss} Ψ^o
tonoplast
cytoskeleton
Direction of Mechanical stress
hypo-osmotic medium
$c^o_{hypo} = c^o_{ss} - \Delta c$
Mucilage swelling due to concentration change
water inflow
$J = Lp\ (\Psi^o + \Delta\Psi - \Psi^i)$
cell swelling
Direction of mechanical stress: increasing
$\Delta\Pi_{hypo}$
PRESSURE SENSOR COMPLEXES ACTIVATED
Extracellular matrix (wall and mucilage) swells
β integrin ?
cytoskeleton ?
mechanosensory channels
DETECTORS
depolarisation
Small Ca^{++} inflow
IP_3 cascade ?
Ca^{++} release from endoplasmic reticulum and vacuole
Ca^{++}-activated Cl^- channels open
K^+ channels open
EFFECTORS
$\Delta\Pi$
Cl^-
K^+
c^i_{hypo}
Finally new steady state is achieved with original turgor $\Delta\Pi$, but new c^i_{hypo} and c^i_{hyper}
hyper-osmotic medium
$c^o_{hyper} = c^o_{ss} + \Delta c$
Hechtian linkage
water outflow
cell shrinkage
$J = Lp\ (\Psi^o - \Delta\Psi - \Psi^i)$
Direction of mechanical stress: decreasing or disappearing
$\Delta\Pi_{hyper}$
??
Inward rectifier activated
H^+ pump activated
Hyperpolarisation
$Cl^-/2H^+$ symport activated ?
$\Delta\Pi$
Cl^-
$2H^+$
H^+
c^i_{hyper}
ADP
ATP
K^+

similar concentrations of K^+ and Ca^{2+} to vacuoles of adjacent cells, although only a small amount of Cl^- was detected (Canny and Huang 1993). A beautiful example of the role of K^+ exchange between symplast and apoplast in controlling cell turgor pressure is found in the "hand-clapping" plant *Desmodium*, whose leaflets show rhythmic movements due to swelling and shrinking of the motor cells of the pulvinus. Antkowiak and Engelmann (1995) used extra- and intracellular electrodes to measure K^+ in the apoplast. They found that when the cells were swollen during the "upstroke", apoplastic K^+ concentration was at its highest and the cells were relatively hyperpolarized. Apoplastic K^+ was at its lowest, and the cells were depolarized, when the cells were shrunken, during the "downstroke".

While changes in concentration of major ions and subsequent changes in $\Delta\Pi$ have been studied extensively, the presence of MS channels is only documented for some of these situations. MS channels are involved in the development of morphogenetic polarity in *Fucus* (Quatrano and Shaw 1997), guard cell physiology (Cosgrove and Hedrich 1991) and algal cell turgor regulation (Beilby and Shepherd 1996; Taylor et al. 1996; Heidecker et al. 1999; Shepherd and Beilby 1999; Shepherd et al. 1999), as well as other phenomena dependent on mechanical, osmotic and surface properties of cells, including embryoid formation from protoplasts (Barthou et al. 1999) and growth of maize callus (Laboure et al. 1999). MS calcium channels appear to be widespread in the epidermis of higher plants, and indeed, MS channels are thought to contribute to differentiation in the majority of plants, since mechanical shear stress on cell walls acts as a positional signal (Ding and Pickard 1993). Mechanical signaling will involve changes in cytoplasmic Ca^{2+} levels (reviewed Haley et al. 1995). Even movements in the wind induce such changes in cytoplasmic Ca^{2+} (Knight et al. 1992). The relationship between stimulus perception, via receptor potentials, and response, that involves regulation of or changes in turgor pressure, is a deeply conserved part of plant development and plant's response to environmental change.

Chains of stimulus and response in touch stimulation

Turgor regulation resulting in movements follows similar sequences even in very different plants. The insectivorous plants such as *Aldrovandra vesiculuosum* (Shimmen 2001a) or the Venus Flytrap (Jacobsen 1965; Benolken and Jacobsen 1970) have been considered in detail. In general terms, bending of a multicellular sensory hair results in receptor potentials, small depolarizations of short duration (usually of the order of 10–15 mV and lasting ~10 s in cells normally having a resting PD between –130 and –200 mV) that are transmitted to motor cells of the trap lobe. These receptor potentials are the "tripwires" for action potentials by depolarizing the membrane to a threshold level that stimulates voltage-gated Ca^{2+} channels to open, and initiate an action potential. If the receptor potential is of critical amplitude, or if receptor potentials come in volleys, and are "summed" (Kishimoto 1968), an action potential is triggered in the trap motor cells. The action potential induces efflux of K^+ and Cl^- and water loss, reducing turgor in motor cells and causing the trap to collapse. Mechanically stimulated receptor potentials have been measured from the early days of research into plant excitability (Pickard 1973). MS channels are responsible for receptor potentials (Shimmen 1996).

The chain from stimulus to response thus consists of:

1. Perception, through *detectors*. Mechanosensory channels are the first detectors of mechanical stimulus. The stimulus is translated from mechanical energy to electrical code (receptor potentials).
2. Transmission. An action potential may be generated in one part of the cell and transmitted along the cell or to several cells.
3. Response, achieved by the *effectors*. Turgor is regulated by controlled efflux of K^+ and Cl^- (activated by Ca^{2+}) and water.

Variations on this theme describe the sequence of the majority of plant responses to mechanical stimulus. However, it is difficult to isolate the behaviour of single cells in multicellular systems. We have aimed to understand the chain of events in single cells, from receptor potentials and action potentials in freshwater Characeae, to turgor regulation in salt-tolerant charophytes.

Charophytes as the ideal experimental system

Complex tissues, where many cells may contribute to the observed electrophysiology, are clearly unsuitable subjects if we want to understand MS channels at the level of single cells. Conversely, protoplasts, in lacking a wall, are perhaps too simple a system when the cell wall and its regulation of turgor pressure is a key factor in the response. Cells of charophytes (especially *Chara*, *Nitella and Lamprothamnium*) are well-explored experimental systems. Studies of charophyte cells have established the fundamentals of plant cell membrane electrophysiology, such as the H^+ translocating ATPase, and plant cell excitability (Shimmen et al. 1994; Wayne 1994). The charophytes have always presented difficulties in terms of classification. They are classified with green algae, yet they form complex enclosed reproductive structures. The chloroplasts of charophytes have grana like those of higher plants but unlike other algae (Gunning and Schwartz 1999). The gain of two chloroplast tRNA introns suggests that charophytes are the green algal ancestors of higher plants (Baldauf et al. 1990; Manhart and Palmer 1990). Sequence data for the Rubisco large sub-unit show that the Characeae are probably monophyletic (McCourt et al. 1996). The organisms express one of the most complex explications of the filamentous

form. Apical cells produce alternating internodal cells and complex nodal aggregations.

Higher plants have charophyte-like ancestors, and extant charophytes probably descended from salt-tolerant ancestors (Winter et al. 1996). The capacity to regulate turgor and tolerate high salinities was probably lost by some charophytes when they successively colonized freshwater niches. The internodal cells of freshwater *Chara* are large single cells (from 7 mm length, in some electrophysiological experiments; up to 36 mm and close to 1 mm in diameter in mechanical stimulation experiments). *Lamprothamnium* has a more variable morphology, and internodes from marine plants are smaller than those in brackish environments (Shepherd et al. 1999). Typically the internodal cells used in electrophysiological experiments, like the ones described here, are about 7 mm long and 0.3 mm in diameter. Internodal cells can be easily excised from the plants and their large size facilitates microelectrode impalements and measurements of action potentials. The large cell size also makes it possible to extract and analyse vacuolar and cytoplasmic contents, or to manipulate the cell compartments by perfusion, permeabilization or centrifugation (Shimmen et al. 1994).

The initial phase of the touch-stimulated action potentials involves first a transitory depolarization (receptor potential) that induces opening of PD-sensitive Ca^{2+} channels, probably in endomembranes (Plieth et al. 1998), and influx of Ca^{2+} to the cytoplasm that can be visualized with aequorin (Williamson and Ashley 1982; Kikuyama and Tazawa 1998). This release is coupled with the sudden cessation of cytoplasmic streaming. Cytoplasmic streaming in the Characeae is dependent on what is normally called "cytoplasmic Ca^{2+} ion concentration" or Ca^{2+}_{cyt} and is motivated by the actin-myosin system. However, Ca^{2+} is actually not mobile in the cytoplasm, in the sense that it does not move by diffusion (Trewavas 1999). Nonetheless, it may make concentration-dependent associations with cytoskeletal and cellular proteins. Ca^{2+} definitely regulates myosin by molecular interactions, since the calmodulin-myosin link is facilitated at low Ca^{2+} concentrations and severed at high concentrations, during the peak of the action potential, when streaming ceases (Yokota et al. 2000).

The touch response in freshwater Characeae

Receptor potentials

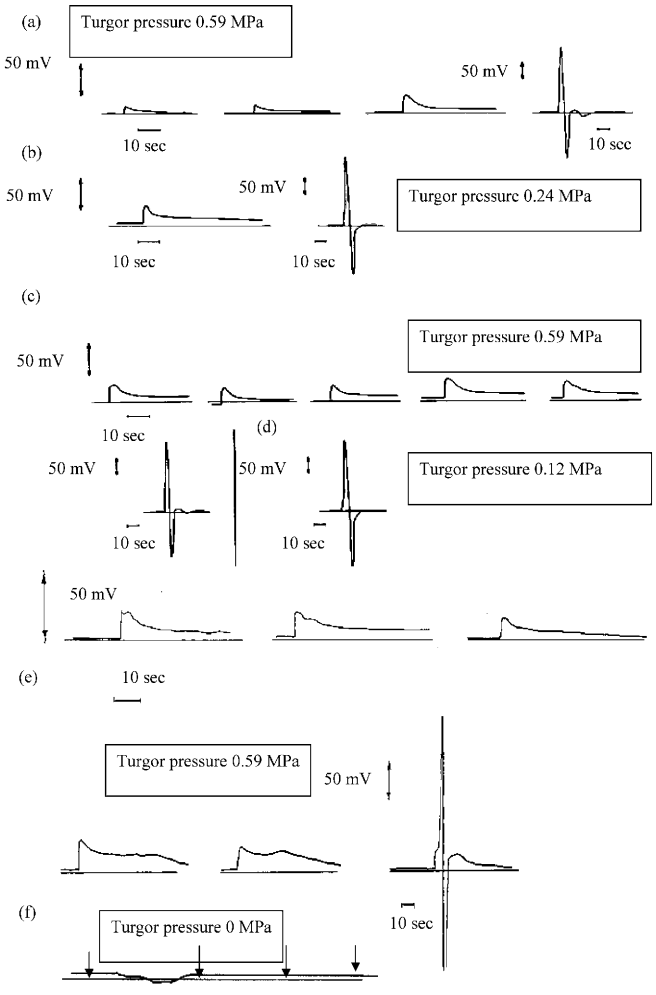
It has been possible to clearly characterize individual receptor potentials in single cells of *Chara*, without competing effects from other cells. Shimmen (1996, 1997a, 1997b, 1997c, 2001a) developed a mechanostimulatory device capable of delivering a reproducible series of mechanical stimuli of increasing energy to single *Chara* cells. The receptor potentials are generated by mechanosensory ion channels, producing transient depolarizations of the cell membrane, whose amplitude varies with stimulus strength, until a threshold depolarization is generated, and the cell fires an action potential (Fig. 2). The receptor potential of critical amplitude, that sufficiently depolarizes the membrane, stimulates an action potential. Below-threshold receptor potentials, fired in succession, can be summed and stimulate action potentials (Kishimoto 1968). Membrane resistance decreases during the RPD and so ion flow must occur, even if its source is difficult to identify. The H^+ pump is not involved in generation of RPDs, nor are they affected by the usual inhibitors of voltage-gated Ca^{2+} and Cl^- channels. The equilibrium potentials of Ca^{2+} and Cl^- across the plasma membrane suggest that these ions are nonetheless involved in generating the RPDs. In experiments where *Chara* cells were stimulated in K^+ solutions with different equilibrium potentials for Cl^-, Cl^- channels appeared to produce the receptor potential (Shimmen 2001a).

Action potentials: multi-functional signals

An action potential is a major event for the cell. The argument that action potentials act as multi-functional signals in plants, through their characteristic release of Ca^{2+} from internal stores into the cytoplasm, was put forward convincingly by Davies (1987). Mechanical information is translated by MS channels into threshold receptor potentials (Shepherd et al. 2001), and a transitory depolarization-induced influx of Ca^{2+} to the cytoplasm follows (Plieth et al. 1998).

It is still controversial whether this Ca^{2+} originates from the external medium (Kikuyama and Tazawa 1998) or from internal stores such as endomembranes (Biskup et al. 1999). Classic experiments (e.g. Findlay 1961; Hope 1961; see also Beilby 1984) showed that the action potential peak was strongly dependent on the external concentration of Ca^{2+}, and that the action potential could be abolished by removing Ca^{2+} from the medium, as was the calcium transient and streaming cessation (Williamson and Ashley 1982). On the other hand, experiments using Mn^{2+} to quench the fluorescence of the Ca^{2+} indicator fura-2 showed that quenching did not occur during an action potential when Mn^{2+} was present in the medium (Plieth et al. 1998). This suggested that Ca^{2+} did not enter from the external medium, if Mn^{2+} is transported similarly to Ca^{2+}.

However, evidence is accumulating to support the theory that Ca^{2+} is released to the cytoplasm from internal stores (Wacke and Thiel 2001). In terms of this theory, depolarization causes elevation of second messenger IP_3 (inositol 1,4,5-trisphosphate) and consequent mobilization of Ca^{2+} from internal stores such as the endoplasmic reticulum (ER; Biskup et al. 1999). The phosphoinositide signalling cascade may be the predominant calcium-regulated second messenger system operating in plants, as argued over a decade ago (Poovaiah et al. 1987). Unlike Ca^{2+}, IP_3 can diffuse in the cytoplasm, which may be how calcium waves are



◀

Fig. 2a–f. Reduction of turgor pressure leads to increased sensitivity to stimulation; conversely, increasing turgor pressure leads to decreased sensitivity to stimulation. The effect on the amplitude and duration of receptor potentials and action potentials of changing turgor pressure is shown in a single cell. Turgor pressures were altered by addition of sorbitol; the cellular osmotic pressure and osmotic pressure of the external solution was measured using a vapor-pressure osmometer. The cells were stimulated by releasing a rod from graded heights, striking the cells with increasing momentum. (**a**) Successive stimuli, *from left to right*, produce receptor potentials of increasing amplitude at the resting turgor pressure of 0.59 MPa. The stimuli had momenta of 5.6×10^{-4}, 8.0×10^{-4} and 9.9×10^{-4} kg m s^{-1}, respectively. The action potential (shown half-size) was stimulated at a momentum of 12×10^{-4} kg m s^{-1}. (**b**) When the cell turgor pressure was decreased to ~0.24 MPa, the cell gave a sub-threshold receptor potential with a far smaller stimulus (5.6×10^{-4} kg m s^{-1}) and fired an action potential at a far smaller stimulus (8.0×10^{-4} kg m s^{-1}). (**c**) Returning the cell turgor pressure to ~0.59 MPa led to a decrease in sensitivity to stimulation. Receptor potentials, *shown left to right*, were given at stimuli with momenta of 5.6×10^{-4}, 8.0×10^{-4}, 9.9×10^{-4}, 12×10^{-4} and 13×10^{-4} kg m s^{-1}, with the action potential firing at 14×10^{-4} kg m s^{-1} (*next line*). (**d**) Sensitivity to stimulation dramatically increased when turgor pressure was reduced to ~0.12 MPa, and the cell fired an action potential with a stimulus of momentum only 5.6×10^{-4} kg m s^{-1}. (**e**) With turgor pressure restored to ~0.59 MPa, the sensitivity to mechanical stimulation again decreased, with the sub-threshold receptor potential firing at a momentum of 13×10^{-4} kg m s^{-1}, and the action potential at a momentum of 14×10^{-4} kg m s^{-1}, as in (**c**). (**f**) When the cell was plamolysed (turgor pressure zero MPa), the cell became unresponsive to stimuli of momenta 5.6×10^{-4}, 8.0×10^{-4}, 9.9×10^{-4} and 12×10^{-4} kg m s^{-1}. Parts (**a**)–(**d**) are adapted from Fig. 3 in Shepherd et al. (2001). Parts (**e**) and (**f**) are from V.A. Shepherd (unpublished results)

propagated (Trewavas 1999). The phosphoinositides are phospholipid monolayers with negative head groups that are integrated with the plasmalemma (Landau and Leshem 1988). A signal activates phospholipase C, an enzyme specific for inositol phospholipids. The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) gives diacylglycerol (DAG) and IP_3. DAG activates protein kinase C and IP_3 induces Ca^{2+} release from the ER (Poovaiah et al. 1987). IP_3 also induces voltage-dependent Ca^{2+} channels in vacuoles to release Ca^{2+} (Alexandre et al. 1990). Microinjection of caged IP_3 can mimic this process by inducing the Ca^{2+} release from internal stores (Thiel et al. 1990). Ca^{2+} is normally maintained at a very low level in the cytoplasm, about 0.1 μM in *Chara* (Williamson and Ashley 1982), whilst the cell wall and internal stores can contain between 0.1 and 10 mM concentrations (Trewavas 1999), and the vacuole and intercellular spaces can contain ~120 mM and ~70 mM Ca^{2+}, respectively (Canny and Huang 1993). During excitation the Ca^{2+} concentration is transiently elevated from 200 nM to 700 nM (Plieth and Hansen 1996). Even minute increases of Ca^{2+} in the cytoplasm have substantial signaling effects.

A major effect of the increase in Ca^{2+} induced by an action potential is the severing of the calmodulin-myosin link (Yokota et al. 2000) and cessation of cytoplasmic streaming. The transient elevation of Ca^{2+} in the cytoplasm activates Cl^- channels (Beilby 1984), accompanied by instantaneous effluxes of Cl^- and K^+ (Oda 1976), a decrease in cell turgor pressure (Zimmerman and Beckers 1978) and transient contraction of the cell (Oda and Linstead 1975).

Direct effects of turgor pressure on receptor potentials; indirect effects on action potentials

In Pickard's model of the plasmalemmal control centre, clusters of MS channels are connected to the cell wall/extracellular matrix by integrin-like proteins that also link with the cytoskeleton (Pickard 1994). Turgor pressure, as the driving force for expansion or contraction of cells, is thus controlled by MS channels linked with the cytoskeleton (Ding and Pickard 1993). This model leads us to expect that receptor potentials should be responsive to changes in cell turgor pressure, but Staves and Wayne (1993) concluded that turgor pressure did not influence the touch-activated action potential in *Chara*. However, they did not consider RPDs, the part of the response actually generated by MS channels.

We found that RPDs in *Chara* are indeed turgor-responsive (Shepherd et al. 2001). Cells experiencing

incipient plasmolysis either lost the ability to respond to mechanical stimulus with receptor potentials, or gave unusual responses (Shepherd et al. 2001; Fig. 2), suggesting that the wall-membrane associations are essential for mechanosensing. When turgor pressure was reduced to a lesser extent, without inducing incipient plasmolysis, the cells gave RPDs of increased amplitude for a given stimulus, relative to the RPD produced at normal turgor pressure. Conversely, when turgor pressure was increased, the cells produced receptor potentials of smaller amplitude for a given stimulus. However, current/voltage analysis showed that this was not due to changes in the membrane PD or the cell conductance, since neither was significantly affected by reduction of turgor. Kiyosawa and Ogata (1987) had shown earlier that the cell electrical resistance was independent of osmotic pressure, but that water permeability decreased when either external or internal osmotic pressure was increased. The enhanced sensitivity to stimulus is due, rather, to changes in the mechanical properties of the cell wall-plasmalemma-cytoskeleton complex, since it can be shown that the force directed radially outwards by the protoplast and the tension of the cell wall both diminish as turgor pressure is decreased (Shepherd et al. 2001). Thus, a given mechanical stimulus is likely to deform the complex more, in less turgid cells, and activate more MS channels.

In keeping with the conclusion of Staves and Wayne (1993), the threshold depolarization for inducing an action potential was unaffected by changes in turgor pressure, and was a voltage-dependent rather than stretch-dependent process. However, the threshold for stimulation of an action potential was reached with a mechanical stimulus of lower energy in cells with reduced turgor. Thus, turgor reduction led to a ''hair-trigger'' touch-response, where sensitivity to touch was greatly increased.

The biological advantages of turgor-dependent receptor potentials

The cell with reduced turgor is more likely to respond to mechanical stimuli with action potentials. We reasoned that such a ''hair-trigger'' RPD in response to stimulus was a defence mechanism, for long-term reduction of turgor signals injury, and turgor reduction can result in inhibition of cell-to-cell communication with neighbouring cells. Plasmodesmata transiently close during an action potential, and normal cell-to-cell communication is not restored until a turgid neighbouring cell is present (Shepherd and Goodwin 1992). Hydrostatic pressure differences across plasmodesmata between adjoining cells can inhibit cell-to-cell transport (Zawadzki and Fensom 1986; Ding and Tazawa 1989). Zawadzki and Fensom (1986) also reported inhibition of ^{14}C transport during prolonged action potentials. The increase in cytoplasmic Ca^{2+} during an action potential appears to transiently but directly affect the permeability of plasmodesmata in *Chara* (Shepherd and Goodwin 1992). This regulation may be brought about by Ca^{2+}-mediated contraction of centrin (Blackman et al. 1999) and is inseparable from the effect of the turgor decrease that can provoke an action potential (Zimmermann and Beckers 1978). Permanent loss of turgor constitutes the ''death signal'' that is transmitted to neighbouring cells (Shimmen 2001b). The cell that loses turgor is ultimately disconnected from the body of the plant, and falls to the substrate in which the plant is growing, taking with it the totipotent node cells that will regenerate new plants. In higher plants, regulated changes in turgor pressure are involved in regulation of complex movements and in development. The Ca^{2+}-regulated touch response in *Chara* shows us how mechanical signals are transduced, in primal form.

Detectors and effectors of osmotic stress: turgor regulation in *Lamprothamnium*

The mechanical stimuli delivered to *Chara* by a mechanostimulatory device (Shimmen 1996) strike the cell and deform the wall and membrane inwards, opposing the force due to hydrostatic pressure ΔP that is directed outwards by the protoplast against the wall (see Fig. 1), which equates to the ΔP multiplied by the cell surface area. By contrast, osmotic stress increases or decreases ΔP directly by swelling or shrinking of the cell, due to water flow induced by decreasing or increasing the external solute concentration (see Fig. 1).

Hypotonic turgor regulation in *Lamprothamnium*

Cells of the salt-tolerant charophyte genus *Lamprothamnium* have the capacity to respond to osmotic shocks with a very extended period of tightly controlled ion efflux or influx, that ultimately allows them to retain their turgor pressure in the face of osmotic and salinity changes. These may be hypotonic shocks, which occur when it rains in the shallow estuarine pools, or hypertonic stress, when the pool evaporates. The hypotonic response is so well researched that by 1990 it had become a model for turgor regulation in salinity-tolerant cells (Okazaki and Tazawa 1990). The continued development of this model will be discussed in this section.

When *Lamprothamnium* cells are exposed to a sudden decrease in osmotic pressure and ion concentration (by say 1/2), a predictable sequence of events occurs (Beilby and Shepherd 1996; Shepherd et al. 1999):

1. An influx of water occurs, the rate of which depends on *Lp* for that cell (Shepherd and Beilby 1999).
2. The membrane PD depolarizes, by 30 to 50 to 100 mV, depending on the original salinity and *Lp* of the cells.

3. Ca^{2+} influxes into the cytoplasm, affecting cytoskeletal proteins of the actin/myosin system, and cytoplasmic streaming stops.
4. The membrane conductance increases up to 10-fold due to Cl^- efflux, from Ca^{2+}-activated Cl^- channels.
5. The Cl^- conductance declines as the K^+ conductance peaks, at about 40 min, with the membrane PD staying close to E_K.
6. After about an hour, cell conductance is reduced to less than the resting level.
7. The "fine tuning" of turgor regulation continues for many hours (Beilby et al. 1999).

Detectors of the hypotonic response

Following pharmacological dissection of the channels involved, we modified the early model of Okazaki and Tazawa (1990), who had described the sequence of events as a negative feedback loop that returned the cell to a "pre-set" turgor level. We showed that the initial depolarization (of about 50 mV) still occurred with K^+ or Ca^{2+} (and consequently Cl^-) channels inhibited, using tetraethylammonium (TEA) and La^{3+} respectively, and that the cell conductance actually declined transiently in the initial phase (Beilby and Shepherd 1996). Furthermore, the time-courses of the Cl^- or K^+ conductances were the same when the respective inhibitors were used, showing that, once triggered, these effluxes continued without regard for re-establishing a pre-set turgor level. We attributed the initial depolarization to the activity of MS channels (Beilby et al. 1999; Shepherd and Beilby 1999; Shepherd et al. 1999; Beilby and Shepherd 2001a), which were hardly affected by inhibitors of voltage-dependent channels in *Chara* (Shimmen 2001a). Thus, the major detector of hypo-osmotic shock in *Lamprothamnium* may be MS channels that produce an extended depolarization resembling a drawn-out receptor potential.

The role of extracellular mucilage

Earlier research by others and ourselves had concentrated on cells living in brackish water (about 1/3 seawater), but we then conducted an ecophysiological study of the algae living in a range of environments, including full seawater. Surprisingly, cells in full seawater could endure equally large or larger hypotonic shocks (from full to 1/2 or 1/4 seawater), but the pattern of hypotonic response resembled that of the brackish-water cells with Ca^{2+} channels inhibited. The cells initially depolarized by a small amount (~20 mV), but the influx of Ca^{2+}, if it occurred at all, was insufficient to halt cytoplasmic streaming. The cell conductance increased only about 1.2-fold beyond the resting level, instead of by 10-fold, indicating that the Ca^{2+}-activated Cl^- channels had largely failed to open. However, the cells maintained their initial depolarization, with current/voltage profiles characteristic of the K^+ state (where the membrane electrical properties are dominated by the large conductance K^+ channels), and the cell conductance was progressively reduced over an hour until it was less than the resting conductance, as we would have expected in the more dilute solution.

A major difference between the brackish-dwelling and marine *Lamprothamnium* cells lay in the distribution of an extracellular mucilage that we identified, using histochemical stains Alcian Blue and Toluidine Blue at low pH, as a mixture of sulfated and carboxylated polysaccharides (Shepherd et al. 1999). The thickness of the mucilage layer was directly related to the salinity of the environment. Cells from plants living in dilute seawater ($< 1/3$) produced little or no mucilage, whilst marine cells produced mucilages up to ~45 μm thick, a very substantial layer in cells that were normally 300 μm in diameter. We concluded that this mucilage, which is chemically similar to other highly charged polyanions like heparin, both delayed the impact of osmotic shocks and had the capacity for binding cations, especially Na^+ and Ca^{2+}. Under different circumstances the mucilage might preferentially bind either Na^+ or Ca^{2+}, as occurs in a proteoheparan sulfate that functions as a blood flow sensor (Siegel et al. 1996). The *Lp* of the cells was directly related to mucilage thickness, and marine cells had an $Lp \approx 3$ times less than that of freshwater *Chara*.

We attributed the initial depolarization of mucilage-coated cells to MS channels, and concluded that the turgor regulation was achieved largely by depolarization-activated K^+ channels, with a relatively small conductance attributable to Cl^- channels. We hypothesized that the MS channels may be coupled with integrin-like protein linkages made with the extracellular matrix, including the mucilage. Enzymatic digestion of the mucilage with heparinase (Shepherd and Beilby 1999) dramatically changed the response to hypotonic shock. Mucilage-coated control cells responded to hypotonic shock with a small depolarization, uninterrupted streaming, and a mere 1.2-fold increase in cell conductance, that declined, after an hour, to less than the resting value in seawater. In contrast, heparinased cells responded to the same hypotonic shock with a large depolarization (sometimes to positive values), a prolonged cessation of cytoplasmic streaming, and a 100-fold (or more) increase in cell conductance. The enormous conductance did not decline in the same time interval as in controls. Although the cells recovered cytoplasmic streaming, showing that Ca^{2+} had been sequestered by the vacuole and/or ER, they retained zero or even positive PDs for several hours. The mucilage sheath remained loosely in place as an unstirred layer, but it lost the capacity for staining at low pH, indicating that the charged structure of the sulfated polysaccharides had been disrupted. Heparinased cells recovered 24 h after being removed from heparinase, and the extracellular mucilage again stained at low pH, suggesting that the charged structure had been recovered. These

cells then responded to a further hypotonic shock similarly to controls.

Modeling the current-voltage (I/V) characteristics of membrane transporters

The detailed electrical behavior of both detector and effector transporters can be studied through modeling the current-voltage (I/V) characteristics in steady state and at the time of osmotic stress. The most conductive membrane transporters, such as the proton pump, the large conductance K^+ channels and the inward and outward rectifiers, have been modeled for charophyte cells employing the "two state" HGSS model (Hansen et al. 1981; Beilby and Walker 1996) and the Goldmann-Hodgkin-Katz (GHK) equation (Beilby and Shepherd 2001a, 2001b). Thus, the current through the proton pump, i_p, is given by Eq. (1):

$$i_p = zFN\frac{k_{io}\kappa_{oi} - k_{oi}\kappa_{io}}{k_{io} + k_{oi} + \kappa_{io} + \kappa_{oi}} \quad (1)$$

where:

$$k_{io} = k_{io}^{o} e^{\frac{zFV}{2RT}} \quad (2)$$

and:

$$k_{oi} = k_{oi}^{o} e^{-\frac{zFV}{2RT}} \quad (3)$$

The F, R, T symbols have their usual meanings; z is the pump stoichiometry, which has been set to 1; N is a scaling factor (2×10^{-8}) and V is the PD across the membrane or membranes. The number of carrier states was reduced to two with a pair of PD-dependent constants, k_{io} and k_{oi}, with a symmetric Eyring barrier (Eqs. 2 and 3), and PD-independent rate constants, κ_{io} and κ_{oi}.

The current through the large conductance K^+ channel, i_K, is modeled by Eq. (4):

$$i_K = \frac{P_{o+}P_{o-}N_K P_K F^2 V\left([K^+]_i - [K^+]_o e^{-\frac{FV}{RT}}\right)}{RT\left(1 - e^{-\frac{FV}{RT}}\right)} \quad (4)$$

where $[K^+]_o$ and $[K^+]_i$ are the K^+ concentrations in the medium and the cytoplasm, respectively. $N_K P_K$ represents the number of K^+ channels and their permeability. It is treated as a single parameter. The PD dependence of the channel opening is simulated by Boltzmann distribution at more negative and more positive PDs, P_{o+} and P_{o-}, given by Eqs. (5) and (6):

$$P_{o+} = 1 - \frac{1}{1 + e^{\frac{z_g F(V - V_{50})}{RT}}} \quad (5)$$

$$P_{o-} = \frac{1}{1 + e^{-\frac{z_g F(V - V_{50})}{RT}}} \quad (6)$$

where z_g is the number of gating charges, V_{50-} and V_{50+} are the half-activation potentials, V_{50}, at the negative and positive PD of channel closure. i_K diminishes at both negative and positive PDs, requiring both P_{o+} and P_{o-}. The inward and outward rectifiers, i_{irc} and i_{orc}, are also modeled by Eq. (4), but with a single probability distribution P_{o+} (Eq. 5) for i_{irc} and P_{o-} (Eq. 6) for i_{orc}.

In cells where the proton pump and i_K have been inhibited by various conditions, the background current, $i_{background}$, can be observed. The background current is modeled by an empirical equation (7) with PD-independent conductance, $g_{background}$, and reversal PD, $V_{background}$, as the ions carrying it have not been identified:

$$i_{background} = g_{background}(V - V_{background}) \quad (7)$$

Figure 3 demonstrates data modeling for a salt-tolerant charophyte *Lamprothamnium* cell, which was transferred from brackish medium [artificial ditch water (ADW) in mM: NaCl 120; KCl 2.8; $CaCl_2$ 2.4; $NaHCO_3$ 0.24; pH 7.6] to quarter of the ionic strength, but with the osmolarity kept constant using sorbitol. In the steady state the large conductance K^+ channels were open (I/V data points and fitted current are shown in gray in part a, i_K in part b, total conductance in part c and $i_{background}$ in part d). The changes in the I/V characteristics are followed as a function of time in quarter ionic strength medium: 5.5 min (short dashing); 12 min (long dashing); 27 min (unequal dashing); 66 min (thin continuous line); 117 min (thick continuous line). The fit parameters are shown in Table 1. Both i_K and $g_{background}$ diminished in medium of lower ionic strength. The streaming speed was not affected. Note that $V_{background}$ changed only slightly throughout this period. These trends can be compared to a cell exposed to a hypotonic shock by halving both the ionic strength and osmolarity of the medium (see fig. 7 in Beilby and Shepherd 2001b). This cell displayed an increase in i_K and $g_{background}$ and a transient depolarization of $V_{background}$ by ~60 mV (the Ca^{2+} channels were blocked by exposure to $LaCl_3$, inactivating Cl^- channels). Thus, the activation of i_K throughout the hypotonic response could be separated from the Cl^- channels (Beilby and Shepherd 1996). As can be seen in Fig. 1, the hypotonic cell experiences water inflow and stretching of the wall and membrane, while the cell in changed ionic medium does not. The MS channels, therefore, are expected to contribute *transiently* to the conductance of the hypotonic cell, but not to the conductance of the cell exposed to lower ionic strength.

The hypothesis that $i_{background}$ flows through the MS channels is examined below.

The I/V characteristics of the MS channels in the salt-tolerant charophyte *Lamprothamnium*

In Fig. 4(a), the data from a mucilaginous *Lamprothamnium* cell (adapted from Fig. 6 of Shepherd et al. 1999) exhibit a mainly linear background current

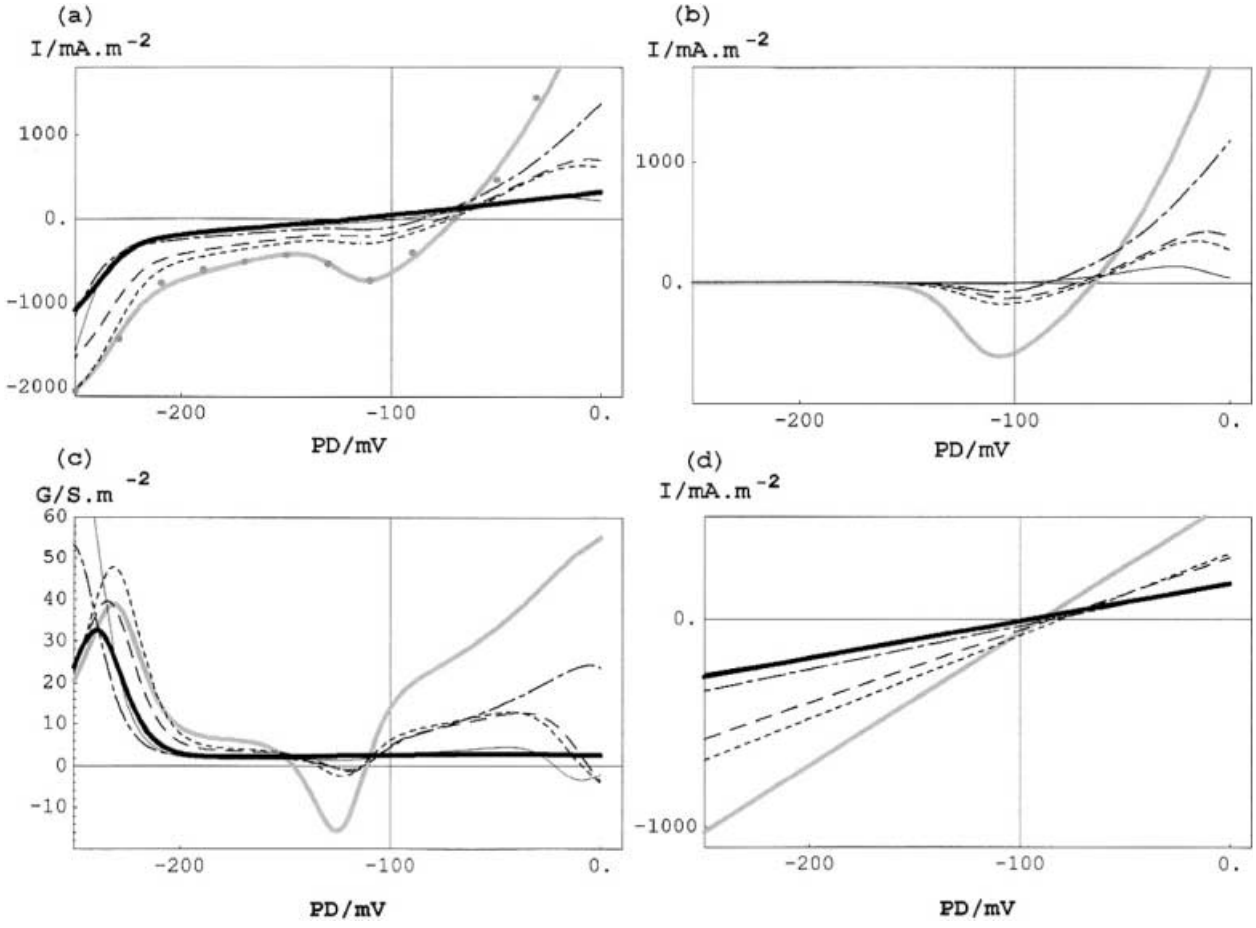


Fig. 3a–d. *I/V data* (*gray points*) obtained from a *Lamprothamnium* cell in equilibrium with ADW (see text for ion composition). The model total current is shown as *thick gray line* in part (**a**). The changes in the *I/V* characteristics are followed as a function of time in quarter ion strength medium: 5.5 min (*short dashing*); 12 min (*long dashing*); 27 min (*unequal dashing*); 66 min (thin continuous line); 117 min (*thick continuous line*). The fit parameters are given in Table 1. The modeled i_K is shown in (**b**), the total *G/V* curves in (**c**) and modeled $i_{background}$ in (**d**). Note that i_K disappeared at 117 min (**b**) and $i_{background}$ has not changed from 66 to 117 min. The cell was not voltage-clamped to sufficiently positive PDs to observe the outward rectifier (Data from M.J. Beilby and V.A. Shepherd, unpublished)

throughout the hypotonic response. The thick gray line shows the steady state in 1/2 SW (seawater), profile 1 after 5 min exposure to 1/4 SW, profile 2 after 7.5 min in 1/4 SW, profile 3 after 30 min in 1/4 SW and profile 4 after 1 h in 1/4 SW. By that time the cell recovered the pre-hypotonic resting PD. This cell exhibited only a small conductance increase. Figure 4(b) is adapted from Fig. 7 in Beilby and Shepherd (2001b). This cell was in the K^+ state in 1/3 SW and the hypotonic challenge was provided by exposure to 1/6 SW. The fitted $i_{background}$ exhibited a transient depolarization of $V_{background}$ and an increase in $g_{background}$ similar to that in Fig. 4(a). Figure 4(c) is adapted from Fig. 6 in Beilby and Shepherd (2001a). The profiles 1–4 represent the $i_{background}$ fitted to populations of cells adapted to a range of salinities: 1, 0.2 SW; 2, 0.4 SW; 3, 0.5 SW; and 4, full SW. These cells face increasing ionic strength media (in mM, Na^+: 77–350; K^+: 2–16; Cl^-: 86–400) and increasing osmolarity (in mosmol kg^{-1}: 140.5–1072), but their vacuolar concentrations had adjusted (see Fig. 1) to achieve the set point turgor of ~300 mosmol kg^{-1} (Bisson and Kirst 1980). Note that while $g_{background}$ increases by an order of magnitude in high ionic strength environment, $V_{background}$ stays remarkably constant. This finding is confirmed in Fig. 4(d), which has been replotted from the previous figure for comparison. As the ionic strength decreases, $g_{background}$ diminishes. As the cytoplasmic concentrations of K^+, Na^+ and Cl^- are thought to remain approximately constant throughout the salinity range (Okazaki et al. 1984), $i_{background}$ would exhibit changes in the reversal PD if it was carried predominantly by any of the above ions. Yao and Bisson (1993) suggest that proton permeability increases in another salt-tolerant charophyte, *Chara longifolia*. This hypothesis is yet to be tested in *Lamprothamnium*. At least some of the conductance increase is likely to arise from electrostatic screening by impermeant ions at the mouth of the channels (Lauger 1976).

Identity of $i_{background}$

Some insight into the identity of $i_{background}$ comes from the measurement of Na^+, K^+ and Cl^- in the

Table 1. Parameters fitted to data from cells exposed to a change to ADW of the same osmotic pressure, but lower ionic strength. The K^+ concentration of the low ion ADW was 0.7 mM, but this concentration was only perceived by the membrane after more than 27 min exposure. The cell PD was not clamped to sufficiently positive levels to activate the outward rectifier i_{orc}

$[K^+]_o$ (mM)	K^+ current					i_{irc}			i_{orc}			$[K^+]_i$ (mM)	$g_{background}$ (S m^{-2})	$V_{background}$ (mV)	E_K (mV)	E_R (mV)
	$N_K P_K$ (×10^{-7} m^3 s^{-1})	V_{50+} (mV)	V_{50-} (mV)	z_{g+}	z_{g-}	$N_K P_K$ (×10^{-7} m^3 s^{-1})	V_{50} (mV)	z_g	$N_K P_K$ (×10^{-9} m^3 s^{-1})	V_{50} (mV)	z_g					
2.8	7.4	> 50	–122	2	3	4.1	–230	3				34.5	6.4	–90	–63	–70
Low ion ADW; 5.5 min: 2.5	2.6	–8	–119	2	3	6.2	–230	3				35.0	4.0	–80	–66	–71
12 min: 2.1	2.6	–1	–115	2	3	6.0	–233	3				34.5	3.5	–85	–71	–76
27 min: 1.2	3.8	–30	–113	3	3	14.0	–250	3				34.5	2.1	–85	–85	–85
66 min: 0.7	1.0	–15	–115	3	3	39	–250	3				34.5	1.7	–95	–98	–96
117 min: 0.7						15.0	–238	3				34.5	1.8	–95	–98	–116

vacuole of *Lamprothamnium* cells from different saline environments and mucilage thickness. Beilby et al. (1999) found that in young cells with thin mucilage, most of hypotonic vacuolar adjustment of these ions occurred within 1 h. The opening of the large conductance K^+ channels and Ca^{2+}-activated Cl^- channels were observed on the same cell population. Further slow adjustment continued for at least 24 h. Older cells with thick mucilage did not exhibit large conductance increases (see the data in Fig. 4a), and regulation took longer than 24 h. However, in both cases, Na^+, K^+ and Cl^- effluxed slowly from the cell without the large conductance channels (K^+ and Cl^-) opening. It is therefore likely that the MS channels are not very selective, or there may be populations of MS channels specific to particular ion species, as found in guard cells (Cosgrove and Hedrich 1991). Why $V_{background}$ changes upon hypotonic exposure remains to be determined. Perhaps the ratio of different types of MS channels alters, or some ions pass through more readily.

Modeling the effects of the mucilage

Figure 3 and Table 1 contain further important information. The mucilage of this cell was 24 μm thick. The best model fit to the data was achieved by slowly decreasing $[K^+]_o$ until the membrane "saw" the external concentration of 0.7 mM at times between 27 and 66 min in low ion ADW. The decrease in $g_{background}$ also followed the time dependence of g_K. This effect of the mucilage as an unstirred layer and ion exchanger is documented from other experiments. Beilby and Shepherd (1996) found that young *Lamprothamnium* cells with thin or no mucilage responded immediately to TEA by hyperpolarization if they were in the K^+ state. Cells with thick mucilage (16.8 μm), on the other hand, took 30 min to respond (Shepherd et al. 1999). As the mucilage thickness also decreases *Lp* (Shepherd et al. 1999), the total effect is to provide a buffer for sudden large changes. Either the slow rate of initial depolarization, due to the MS channels, fails to activate the Ca^{2+} channels, or the inflow of Ca^{2+} is slow enough for the re-sequestration to keep the cytoplasmic concentration down. Thus streaming is not affected and Ca^{2+}-activated Cl^- channels fail to open. A similar effect is observed for the action potential, when the electrical stimulus is applied as slow depolarization (Fujita and Mizuguchi 1955).

The elimination of the large conductance K^+ channels from the hypotonic effect was observed for some older cells with thick mucilage (> 20 μm; Shepherd et al. 1999). This effect might arise from the reduced *Lp* and consequently smaller water inflow upon the hypotonic challenge. The modeling of the hypotonic response of cells with no mucilage indicates an immediate rise in $[K^+]_{cyt}$ upon exposure to hypotonic media (Beilby and Shepherd 2001b), which precedes the activation of i_K. A

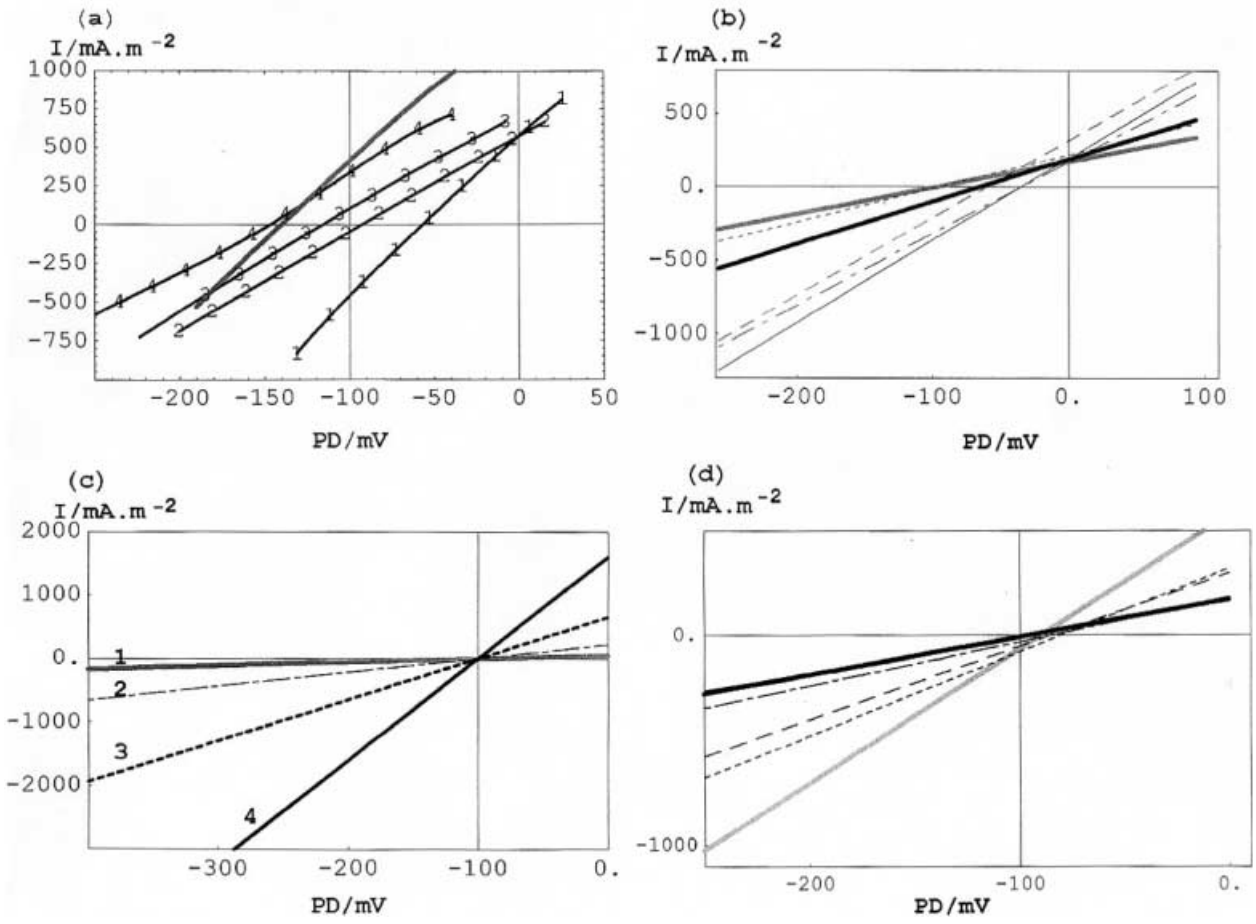


Fig. 4a–d. Comparison of observed and modeled $i_{background}$ under a range of conditions. (**a**) Data from a mucilaginous *Lamprothamnium* cell, which exhibited a mainly linear background current throughout the hypotonic response. The *thick gray line* shows the steady-state I/V profile in 1/2 seawater (SW), which contains some contribution from the proton pump as the resting PD is hyperpolarized. *Profile 1* was measured after 5 min exposure to 1/4 SW, *profile 2* after 7.5 min in 1/4 SW, *profile 3* after 30 min in 1/4 SW and *profile 4* after 1 h in 1/4 SW. Adapted from Fig. 6 in Shepherd et al. (1999). (**b**) The fitted $i_{background}$ exhibited transient depolarization of $V_{background}$ and increase in $g_{background}$ similar to that in (**a**). The cell was in the K^+ state in 1/3 SW (*thick gray line*) and the hypotonic challenge was provided by exposure to 1/6 SW. The changes in the I/V characteristics are followed as a function of time in 1/6 SW: 3.0 min (*short dashing*); 10 min (*long dashing*); 15 min (*thin line*); 22 min (*unequal dashing*); 85 min (*thick line*). The fit parameters are given in Beilby and Shepherd (2001b). Adapted from Fig. 7 in Beilby and Shepherd (2001b). (**c**) The fitted $i_{background}$ is adapted from Fig. 6 in Beilby and Shepherd (2001a). The *profiles 1–4* represent the $i_{background}$ fitted to populations of cells adapted to a range of salinities: *1*, 0.2 SW; *2*, 0.4 SW; *3*, 0.5 SW; *4*, full SW. These cells face increasing ionic strength media (in mM, Na^+: 77–350; K^+: 2–16; Cl^-: 86–400) and increasing osmolarity (in mosmol kg^{-1}: 140.5–1072), but their vacuolar concentrations had adjusted (see Fig. 1). (**d**) The fitted $i_{background}$ is re-plotted from the previous figure for comparison. The different lines are as in Fig. 3

possible scenario involves water inflow into the vacuole, MS channels on the tonoplast allowing inflow of K^+ into the cytoplasmic compartment, and activation of the large conductance K^+ channels. A slower water inflow might not produce sufficient $[K^+]_{cyt}$ rise and the K^+ outflow is then mediated by the plasmalemma MS channels alone.

Hypertonic regulation

Hypertonic turgor regulation in *Lamprothamnium* is quite different from hypotonic regulation. The effectors of this response are the proton pump that hyperpolarizes the cell PD, the inward K^+ rectifier that transports K^+ into the cell, and probably the $2H^+/Cl^-$ symporter, transporting Cl^- into the cell. This type of symporter was described in *Chara* (Beilby and Walker 1981).

The detectors of the hypertonic stress are not known and MS channels are probably not involved. However, cells under extreme hypertonic stress plasmolyse, and can potentially supply information about the structural attachments of the MS channels by revealing wall-membrane linkages or Hechtian strands.

Effects of plasmolysis

Lamprothamnium cells are difficult to plasmolyse. When plasmolysis is achieved at very low pH (<0.5) in the

presence of the stain Alcian Blue, the stained extracellular sulfated polysaccharide mucilage is clearly seen to make stained unbroken linkages at regular intervals with the cell wall and the plasmalemma (Fig. 5). *Chara* cells lack extracellular sulfated polysaccharides and are easily plasmolysed, in contrast to *Lamprothamnium* cells. Some plasmolysed *Chara* cells retain en bloc connections with the cell wall close to intercellular junctions, rather than the discrete evenly spaced linkages seen in *Lamprothamnium*. *Chara* cells at zero turgor can have either of two different electrophysiological profiles (McCulloch and Beilby 1997): the first (80% of cells) with very large conductance, the second (20% of cells) with relatively normal I/V profiles, derived possibly through the kinds of wall-membrane linkages seen in some plasmolysed *Chara* cells (Fig. 5).

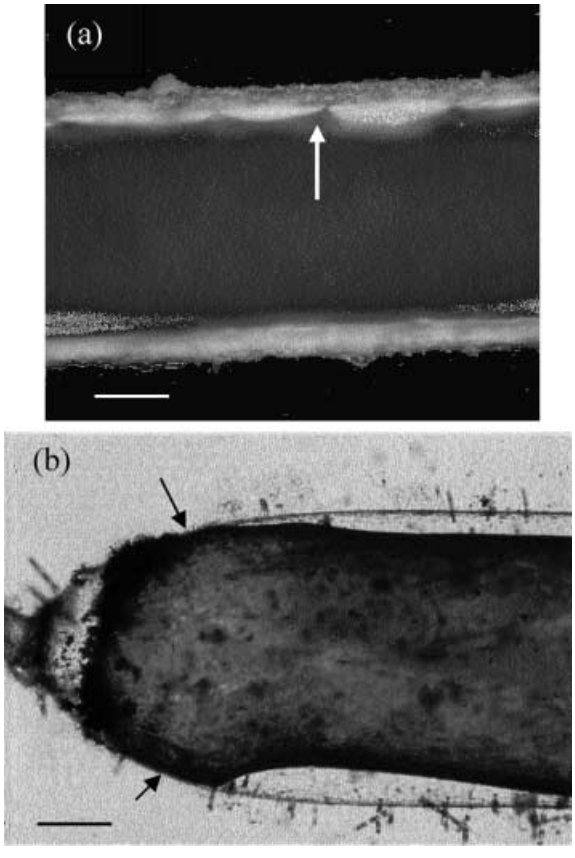


Fig. 5a, b. Comparison of plasmolysis in *Lamprothamnium* and *Chara*. (**a**) Plasmolysed cell of salt-tolerant *Lamprothamnium*, stained with Alcian Blue at pH 0.5 to stain sulfated polysaccharides (sulfates are the only groups in these polysaccharides that are still ionized at this pH). Note the thick layer of extracellular mucilage. Plasmolysis reveals regularly spaced links between the extracellular mucilage, cell wall, and the plasma membrane (*arrow*). Scale bar: 100 μm. Adapted from Fig. 8h in Shepherd et al. (1999). (**b**) Plasmolysed cell of freshwater *Chara*. The extracellular mucilage is absent. The protoplast forms en bloc linkages with the cell wall (*arrow*), usually near the cell ends. However, the regularly spaced linkages with the extracellular matrix, seen above, are absent. *Scale bar*: 100 μm

Conclusion

The capacity for mechanosensing in charophytes is a primal response to touch and osmotic shocks. The striking similarities between the response to hypotonic shock in *Lamprothamnium* and bovine lens cells, both experiencing efflux of Cl^-, K^+ and volume/turgor restoration, show how fundamental such mechanisms are to cell behaviour. In this sense, *Chara*, with its dilute environment, is the odd one out, whilst mammalian cells and *Lamprothamnium* share certain adaptations to a salty, marine world. Ancient, fundamental cellular mechanisms, such as mechanosensory and other ion channels, evolved in osmoregulating procaryotes (Chamberlin and Strange 1989; Harris-Warwick 2000). Sulfated polysaccharides, like those synthesized by *Lamprothamnium*, have a great capacity to ameliorate the impact of osmotic shocks in marine algae, by functioning as ion exchangers and by absorbing/releasing water. This is demonstrated by their growing role in medical research and industry, where they are used as anticoagulants for blood (Maeda et al. 1991), anti-HIV chemicals (Beress et al. 1993), to inhibit the growth of cancer cells and as gels in industry (Yaron et al. 1992). We have been able to examine some of the biophysical properties of these gels in vivo, including their capacity to greatly delay the impact of osmotic shocks and ionic shocks and to greatly decrease *Lp*, the hydraulic conductivity, depending on the thickness of the charged layers.

We conclude that MS channels are the first detectors in the line of stimulus perception. In response to touch or a hypotonic shock, they depolarize the cell membrane to critical levels that activate Ca^{2+} channels, initiating the action potential in response to touch, or a prolonged efflux of Cl^- and K^+ in the turgor-regulation response to hypotonic shock in *Lamprothamnium*. Turgor regulation and salinity tolerance depend also on the gel properties of extracellular secreted sulfated mucilages that may interact with MS channels. Turgor regulation is a slow controlled process through which ions are released in successive waves, Ca^{2+} followed by Cl^-, followed by K^+, until the cell reduces its turgor.

The movements of higher plants in response to stimuli can be very complex, including the famous examples of the folding of *Mimosa* leaves or the trap closure in the Venus Flytrap, which operate as "osmotic machines" (Hill and Findlay 1981). Although complex, such behaviour is ultimately attributable to similar processes. Commencing with perception of stimulus strength, the translation and transmission of signals in the form of action potentials leads to loss of K^+ and Cl^- (or other anions) and collapse of turgor. The mechanisms of touch perception and turgor regulation, dissected on the level of a single cell in *Chara* and in *Lamprothamnium*, show us in fundamental form the processes that have been elaborated in higher plants to form these behavioural sequences.

Acknowledgements We gratefully acknowledge funding from the Australian Research Council (grant to M.J.B.), the Japan Society for Promotion of Science and the Australian Academy of Sciences for a travel grant enabling V.A.S. to visit T.S.'s laboratory.

References

Alexandre J, Lassalles JP, Kado RT (1990) Opening of channels in isolated beetroot vacuole membrane by inositol 1,4,5-trisphosphate. Nature 343:567–570

Antkowiak B, Engelmann W (1995) Oscillations of apoplasmic K^+ and H^+ activities in *Desmodium motorium* (Houtt.) Merril. pulvini in relation to the membrane potential of motor cells and leaflet movements. Planta 196:350–356

Baldauf SL, Manhart JR, Palmer JD (1990) Different fates of the chloroplast tufA gene following its transfer to the nucleus in green algae. Proc Natl Acad Sci USA 87:5317–5321

Barthou H, Petitprez M, Briere C, Souvre A, Alibert G (1999) RGD-mediated membrane-matrix adhesion triggers agarose-induced embryoid formation in sunflower protoplasts. Protoplasma 206:143–151

Beilby MJ (1984) Calcium in plant action potentials. Plant Cell Environ 7:415–421

Beilby MJ, Shepherd VA (1996) Turgor regulation in *Lamprothamnium papulosum*. 1. I/V analysis and pharmacological dissection of the hypotonic effect. Plant Cell Environ 19:837–847

Beilby MJ, Shepherd VA (2001a) Modeling the current-voltage characteristics of charophyte membranes. II. The effect of salinity on membranes of *Lamprothamnium papulosum*. J Membr Biol 181:77–89

Beilby MJ, Shepherd VA (2001b) Modeling the current-voltage characteristics of charophyte membranes. III. K^+ state of *Lamprothamnium*. Aust J Plant Physiol 28:541–550

Beilby MJ, Walker NA (1981) Chloride transport in *Chara* membranes. I. Kinetics and current-voltage curves for a probable proton symport. J Exp Bot 32:43–54

Beilby MJ, Walker NA (1996) Modeling the current-voltage characteristics of *Chara* membranes. I. The effect of ATP removal and zero turgor. J Membr Biol 149:89–101

Beilby MJ, Cherry CA, Shepherd VA (1999) Dual turgor regulation response to hypotonic stress in *Lamprothamnium papulosum*. Plant Cell Environ 22:347–359

Benolken RM, Jacobsen SL (1970) Response properties of a sensory hair excised from Venus's Flytrap. J Gen Physiol 56:64–82

Beress A, Wasserman O, Bruhn T, Beress L, Kraiselfburd EN, Gonzalez LV, Demotta GE, Chavez PI (1993) A new procedure for the isolation of anti-HIV compounds, polysaccharides and polyphenols from the marine alga *Fucus vesicolosus*. J Nat Prod 56:478–488

Biskup BD, Gradmann D, Thiel G (1999) Calcium release from IP_3-sensitive stores initiates action potential in *Chara*. FEBS Lett 453:72–76

Bisson MA, Kirst G (1980) *Lamprothamnium*, a euryhaline charophyte. II. Osmotic relations and membrane potential at steady state. J Exp Bot 31:1237–1244

Blackman LM, Harper JDI, Overall RL (1999) Localisation of a centrin-like protein to higher plant plasmodesmata. Eur J Cell Biol 78:297–304

Canny MJ (1990) What becomes of the transpiration stream? New Phytol 114:341–368

Canny MJ (1995) A new theory for the ascent of sap: cohesion supported by tissue pressure. Ann Bot 75:343–357

Canny MJ (1998) Applications of the compensating pressure theory of water transport. Am J Bot 85:897–909

Canny MJ, Huang CX (1993) What is in the intercellular spaces of roots? Evidence from the cryo-scanning microscope. Physiol Plant 87:561–568

Chamberlin ME, Strange K (1989) Anisomotic cell volume regulation: a comparative review. Am J Physiol 257:C159–C173

Cosgrove DJ, Hedrich R (1991) Stretch-activated chloride, potassium and calcium channels coexisting in plasma-membrane of guard cells of *Vicia faba*. Planta 186:143–153

Davies E (1987) Action potentials as multi-functional signals in higher plants: a unifying hypothesis to explain apparently disparate wound responses. Plant Cell Environ 10:623–631

Ding D-Q, Tazawa M (1989) Influence of cytoplasmic streaming and turgor pressure gradient on the transnodal transport of rubidium and electrical conductance in *Chara*. Plant Cell Physiol 30:739–748

Ding JP, Pickard BG (1993) Mechanosensory calcium-selective cation channels in epidermal cells. Plant J 3:83–110

Findlay GP (1961) Voltage-clamp experiments with *Nitella*. Nature 191:812–814

Findlay GP (2001) Membranes and the electrophysiology of turgor regulation. Aust J Plant Physiol 28:617–634

Fujita M, Mizuguchi K (1955) Excitation in *Nitella*, especially in relation to electric stimulation. Cytologia (Tokyo) 21:135–145

Gunning BES, Schwartz OM (1999) Confocal microscopy of thylakoid autofluorescence in relation to origin of grana and phylogeny in the green algae. Aust J Plant Physiol 26:695–708

Haley A, Russell AJ, Wood N, Allan AC, Knight M, Campbell AK, Trewavas AJ (1995) Effects of mechanical signaling on plant cell cytosolic calcium. Proc Natl Acad Sci USA 92:4124–4128

Hamill OP, Martinac B (2001) Molecular basis for mechanotransduction in living cells. Physiol Rev 81:685–740

Hansen U-P, Gradmann D, Sanders D, Slayman CL (1981) Interpretation of current-voltage relationships for "active" ion transport systems. I. Steady-state reaction-kinetic analysis of class-I mechanisms. J Membr Biol 63:165–190

Harris-Warwick RM (2000) Ion channels and receptors: molecular targets for behavioural evolution. J Comp Physiol A 186:605–616

Heidecker M, Wegner LH, Zimmermann U (1999) A patch-clamp study of ion channels in protoplasts prepared from the marine alga *Valonia utricularis*. J Membr Biol 172:235–247

Hill BS, Findlay GP (1981) The power of movement in plants: the role of osmotic machines. Q Rev Biophys 14:173–222

Hope AB (1961) The action potential in cells of *Chara*. Nature 191:811–812

Jacobsen SL (1965) Receptor response in Venus's Flytrap. J Gen Physiol 49:117–129

Kikuyama M, Tazawa M (1998) Temporal relationship between action potential and Ca^{2+} transient in characean cells. Plant Cell Physiol 39:1359–1366

Kinraide TB (2001) Ion fluxes considered in terms of membrane-surface electrical potentials. Aust J Plant Physiol 28:605–616

Kishimoto U (1968) Response of *Chara* internodes to mechanical stimulation. Ann Rep Biol Works Fac Sci Osaka Univ 16:61–66

Kiyosawa K, Ogata K (1987) Influence of external osmotic pressure on water permeability and electrical conductance of *Chara* cell membrane. Plant Cell Physiol 28:1013–1022

Knight MR, Smith SM, Trewavas AJ (1992) Wind-induced plant motion immediately increases cytoplasmic calcium. Proc Natl Acad Sci USA 89:4967–4971

Kwiatkowska M (1988) Symplasmic isolation of *Chara vulgaris* L. antheridium and mechanisms regulating the process of spermatogenesis. Protoplasma 142:137–146

Laboure AM, Faik A, Mandaron P, Falconet D (1999) RGD-dependent growth of maize calluses and immunodetection of an integrin-like protein. FEBS Lett 442:123–128

Landau EH, Leshem YY (1988) Biophysical interactions of membrane anionic phospholipids with pH, calcium and auxins. J Exp Bot 39:1689–1697

Lauger P (1976) Diffusion-limited ion flow through pores. Biochim Biophys Acta 455:493–509

Maeda M, Uehara T, Harada N, Sekiguchi M, Hiraoka A (1991) Heparinoid-active sulphated polysaccharides from *Monostroma nitidum* and their distribution in the Chlorophyta. Phytochemistry 30:3611–3614

Manhart JR, Palmer JD (1990) The gain of two chloroplast tRNA introns marks the green algal ancestors of land plants. Nature 345:268–270

McCourt RM, Karol KG, Guerlesquin M, Feist M (1996) Phylogeny of extant genera in the family Characeae (Charales, Charophyceae) based on rbcL sequences and morphology. Am J Bot 83:125–131

McCulloch SR, Beilby MJ (1997) The electrophysiology of plasmolysed cells of *Chara australis*. J Exp Bot 48:1383–1392

Oda K (1976) Simultaneous recording of potassium and chloride efflux during an action potential in *Chara corallina*. Plant Cell Physiol 17:1085–1088

Oda K, Linstead PJ (1975) Changes in cell length during action potentials in *Chara*. J Exp Bot 26:228–239

Okazaki Y, Tazawa M (1990) Calcium ion and turgor regulation in plant cells. J Membr Biol 114:189–194

Okazaki Y, Shimmen T, Tazawa M (1984) Turgor regulation in brackish charophyte, *Lamprothamnium succinctum*. II. Changes in K^+, Na^+ and Cl^- concentrations, membrane potential and membrane resistance during turgor regulation. Plant Cell Physiol 25:573–581

Pickard BG (1973) Action potentials in higher plants. Bot Rev 39:172–201

Pickard BG (1994) Contemplating the plasmalemmal control center model. Protoplasma 182:1–9

Plieth C, Hansen U-P (1996) Methodological aspects of pressure-loading of fura-2 into characean cells. J Exp Bot 47:1601–1612

Plieth C, Sattelmacher B, Hansen U-P, Thiel G (1998) The action potential in *Chara*: Ca^{2+} release from internal stores visualized by Mn^{2+} – quenching of fura dextran. Plant J 13:167–175

Poovaiah BW, Reddy ASM, McFadden JJ (1987) Calcium messenger system: role of protein phosphorylation and inositol bisphospholipids. Physiol Plant 69:569–573

Quatrano RS, Shaw SL (1997) Role of the cell wall in the determination of cell polarity and the plane of cell division in *Fucus* embryos. Trends Plant Sci 2:15–21

Shepherd VA (1999) Bioelectricity and the rhythms of sensitive plants – the biophysical research of Jagadis Chandra Bose. Curr Sci 77:189–195

Shepherd VA, Beilby MJ (1999) The effect of an extracellular mucilage on the response to osmotic shock in the charophyte alga *Lamprothamnium papulosum*. J Membr Biol 170:229–242

Shepherd VA, Goodwin PB (1992) Seasonal patterns of cell-to-cell communication in *Chara corallina*. 1. Cell-to-cell communication in vegetative lateral branches during winter and spring. Plant Cell Environ 15:137–150

Shepherd VA, Beilby MJ, Heslop DS (1999) Ecophysiology of the hypotonic response in the salt-tolerant charophyte *Lamprothamnium papulosum*. Plant Cell Environ 22:333–346

Shepherd VA, Shimmen T, Beilby MJ (2001) Mechanosensory ion channels in *Chara*: the influence of cell turgor pressure on touch-activated receptor potentials and action potentials. Aust J Plant Physiol 28:551–566

Shimmen T (1996) Studies on mechano-perception in characean cells: development of a monitoring apparatus. Plant Cell Physiol 37:590–597

Shimmen T (1997a) Studies on mechano-perception in Characeae: pharmacological analysis. Plant Cell Physiol 38:139–148

Shimmen T (1997b) Studies on mechano-perception in Characeae: effects of external Ca^{2+} and Cl^-. Plant Cell Physiol 38:691–697

Shimmen T (1997c) Studies on mechano-perception in Characeae: decrease in membrane electrical resistance in receptor potentials. Plant Cell Physiol 38:1298–1301

Shimmen T (2001a) Involvement of receptor potentials and action potentials in mechano-perception in plants. Aust J Plant Physiol 28:567–576

Shimmen T (2001b) Electrical perception of "death message" in *Chara*: involvement of turgor pressure. Plant Cell Physiol 42:366–373

Shimmen T, Mimura T, Kikuyama M, Tazawa M (1994) Characean cells as a tool for studying electrophysiological characteristics of plant cells. Cell Struct Funct 19:263–278

Siegel G, Malmsten M, Wenzel K, Kauschmann A, Kluβendorf D (1996) Physicochemical and medical applications of a blood flow sensor. In: Fane AG (ed) Proceedings of the 1st IMSTEC Conference. UNESCO Centre for Membrane Science and Technology, Sydney, Australia

Srinivas SP, Bonnano JA, Hughes BA (1998) Assessment of swelling-activated Cl^- channels using halide-sensitive fluorescent indicator 6-methoxy-*N*-(3-sulphopropyl)quinolium. Biophys J 75:115–123

Staves MP, Wayne R (1993) The touch-induced action potential in *Chara*: inquiry into the ionic basis and the mechanoreceptor. Aust J Plant Physiol 20:471–488

Taylor AR, Manison NFH, Fernandez C, Wood J, Brownlee C (1996) Spatial organization of calcium signaling involved in cell volume control in the *Fucus* rhizoid. Plant Cell 8:2015–2031

Thiel G, MacRobbie EAC, Hanke DE (1990) Raising the intracellular level of inositol 1,4,5-trisphosphate changes plasma membrane ion transport in characean algae. EMBO J 9:1737–1741

Trewavas A (1999) Le calcium, c'est la vie: calcium makes waves. Plant Physiol 120:1–6

Wacke M, Thiel G (2001) Electrically triggered all-or-none Ca^{2+} liberation during action potential in the giant alga *Chara*. J Gen Physiol 118:11–21

Wayne R (1994) The excitability of plant cells: with a special emphasis on characean internodal cells. Bot Rev 60:265–367

Williamson RE, Ashley CC (1982) Free Ca^{2+} and cytoplasmic streaming in the alga *Chara*. Nature 296:647–651

Winter U, Soulie-Marsche I, Kirst G (1996) Effects of salinity on turgor pressure and fertility in Tolypella (Characeae). Plant Cell Environ 19:869–879

Yao X, Bisson MA (1993) Passive proton conductance is the major reason for membrane depolarization and conductance increase in *Chara buckellii* in high salt condition. Plant Physiol 103:197–203

Yaron A, Cohen E, Arad S (1992) Stabilization of *Aloe vera* gel by interaction with sulphated polysaccharides from red microalgae and with xanthan gum. J Agric Food Chem 40:1316–1320

Yokota E, Muto S, Shimmen T (2000) Calcium-calmodulin suppresses the filamentous actin-binding activity of a 135-kilodalton actin-binding protein isolated from lily pollen tubes. Plant Physiol 123:645–654

Zawadski T, Fensom DS (1986) Transnodal transport of ^{14}C in *Nitella flexilis*. II. Tandem cells with applied pressure gradients. J Exp Bot 37:1353–1363

Zimmermann U, Beckers F (1978) Generation of action potentials in *Chara corallna* by turgor pressure changes. Planta 138:173–179