Mechano-perception in \textit{Chara} cells: the influence of salinity and calcium on touch-activated receptor potentials, action potentials and ion transport

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\textbf{ABSTRACT}

This paper investigates the impact of increased salinity on touch-induced receptor and action potentials of \textit{Chara} internodal cells. We resolved underlying changes in ion transport by current/voltage analysis. In a saline medium with a low Ca$^{2+}$ ion concentration \([\text{[Ca}^{2+}]_{\text{ext}}]\), the cell background conductance significantly increased and proton pump currents declined to negligible levels, depolarizing the membrane potential difference (PD) to the excitation threshold \([\text{action potential (AP)}_{\text{threshold}}]\). The onset of spontaneous repetitive action potentials further depolarized the PD, activating K$^+$ outward rectifying (KOR) channels. K$^+$ efflux was then sustained and irrevocable, and cells were desensitized to touch. However, when \([\text{Ca}^{2+}]_{\text{ext}}\) was high, the background conductance increased to a lesser extent and proton pump currents were stimulated, establishing a PD narrowly negative to \(\text{AP}_{\text{threshold}}\). Cells did not spontaneously fire, but became hypersensitive to touch. Even slight touch stimulus induced an action potential and further repetitive firing. The duration of each excitation was extended when \([\text{Ca}^{2+}]_{\text{ext}}\) was low. Cell viability was prolonged in the absence of touch stimulus. \textit{Chara} cells eventually depolarize and die in the saline media, but touch-stimulated and spontaneous excitation accelerates the process in a Ca$^{2+}$-dependent manner. Our results have broad implications for understanding the interactions between mechano-perception and salinity stress in plants.

\textit{Key-words:} excitation; plant mechano-perception; proton pump; salt stress.

\textbf{INTRODUCTION}

The sometimes-dramatic responses of plants to touch have intrigued biologists for over a century. A voluminous literature is devoted to the well-known thigmotactic behaviours of \textit{Mimosa}, the Venus’s Flytrap \textit{Dionaea}, the bladderwort \textit{Aldrovanda} (Sibaoka 1991; Shimmen 2001) and the trigger-plant \textit{Stylidium} (Hill & Findlay 1981; Findlay 1984). In recent years, it has become clear that the capacity for perceiving and responding to mechanical stimuli is also fundamental to subtle plant behaviours including responses to gravity, temperature, environmental osmolarity, and turgor-controlled growth and development (reviewed by Fasano, Massa & Gilroy 2002; Jaffe, Leopold & Staples 2002; Baluska \textit{et al.} 2003; Telewski 2006; Haswell 2007; Pickard 2007).

It is thought that mechano-perception in plant cells commences with membrane deformation, whether this occurs directly, or indirectly, via transmembrane protein tethers coupled to the cytoskeleton and the cell wall (reviewed by Fasano \textit{et al.} 2002). Specifically, force perception translates into the activity of mechano-sensory Ca$^{2+}$ ion channels, modulated in a multitude of ways accounting for multiple types of response (Pickard 2007). Mechano-perception rests upon Ca$^{2+}$-dependent second messenger systems, and cytoplasmic Ca$^{2+}$ is elevated following different kinds of mechanical stimulus (Haley \textit{et al.} 1995). In Trewavas’ model (Trewavas 1999a,b), each kind of stimulus elicits a unique Ca$^{2+}$ signal with distinct topology.

The electrophysiology of mechano-perception is difficult to study in small cells of complex tissues. Giant internodal cells of the Charales have been widely used to research mechano-perception at the cellular level.

Embryophytes (land plants) are often viewed as being synonymous with the entire plant kingdom. Phylogenetically, the Charales are the extant sister group to all embryophytes, with which they share some fundamental biochemical and physiological processes (McCourt, Delwiche & Karol 2004). These processes include aspects of excitability, membrane transport (Tazawa & Shimmen 2001) and mechano-perception (Shimmen 2001).

In giant cells of the Charales, mechanical stimuli producing compressive or decompressive mechanical stress on the cell wall deform (Iwabuchi, Kaneko & Kikuyama 2005) or alter the tension (Iwabuchi, Kaneko & Kikuyama 2008) of the cell membrane, activating mechano-sensory ion channels. These channels transduce the touch stimulus into an electrical signal or receptor potential (Shimmen 1996, 1997a,b,c). The receptor potential is a small depolarization brought about by mechano-sensory Ca$^{2+}$ channels at
the plasma membrane. The Ca$^{2+}$ released stimulates Ca$^{2+}$-activated Cl$^{-}$ channels (Kaneko et al. 2005), producing a Cl$^{-}$ efflux (Shimmen 1996, 1997a,b,c). The receptor potential increases in amplitude and/or duration more or less incrementally with increasing magnitude of stimulus (Kishimoto 1968; Shimmen 1996; Kaneko et al. 2005) until a critical threshold voltage initiates an action potential (reviewed by Beilby 2007). In contrast to the action potential, the receptor potential does not propagate intercellularly.

An action potential involves a significant elevation of cytoplasmic Ca$^{2+}$ from 200 to 700 nm (Pließ & Hansen 1996). The elevated Ca$^{2+}$ activates Cl$^{-}$ efflux via Ca$^{2+}$-activated Cl$^{-}$ channels, further depolarizing the cell and activating outwardly directed voltage-dependent K$^{+}$ channels (reviewed, Beilby 2007). Under normal conditions, the action potential has a characteristic threshold and form, and propagates from cell to cell (Beilby 2007). The result is an efflux of Cl$^{-}$ and K$^{+}$ (Oda 1976; Beilby 1984, 2007; Wayne 1994), water efflux and transient turgor reduction (Zimmermann & Beckers 1978), and cell contraction (Oda & Linstead 1975). The motif of elevated cytoplasmic Ca$^{2+}$, efflux of Cl$^{-}$ and K$^{+}$, and turgor loss and contractility, is elaborated into turgor-based osmotic machinery that enables a wide repertoire of mecano-responses in land plants (Hill & Findlay 1981).

We established that cell turgor pressure modulates mechano-perception in Chara internodal cells (Shepherd, Shimmen & Beilby 2001; Shepherd, Beilby & Shimmen 2002). The critical receptor potential, which triggers an action potential, occurs with weaker stimulus when turgor pressure is decreased (Shepherd et al. 2001, 2002). Only the receptor potential is affected. The voltage threshold for initiating an action potential is unchanged (Staves & Wayne 1993; Shepherd et al. 2001). As discussed earlier, the receptor potential is the electrophysiological manifestation of mechano-perception. Furthermore, it is a change in turgor pressure, rather than its absolute magnitude, that elicits sensitization to mechanical stimulus (Shepherd et al. 2001).

The ability to regulate cell volume and/or turgor pressure is fundamental to the lives of cells. Action potentials probably evolved from osmo-regulatory processes of ancient bacteria inhabiting environments of variable salinity (Gradmann & Mumert 1980). The responses of charophyte cells to changes in environmental osmolarity and salinity involve mechano-perception (Shepherd et al. 2002). This may extend to other plant cells. For example, Arabidopsis leaf cells respond to hypotonic or hypertonic media with Ca$^{2+}$ transients, mediated by stretch-activated (or mechanosensory) channels (Hayashi et al. 2006), and increased salinity also elevates cytoplasmic Ca$^{2+}$ in Arabidopsis cells (Knight, Trewavas & Knight 1997).

Considering that Chara cells become increasingly sensitive to mechanical stimulation as turgor decreases (Shepherd et al. 2001), it is probable that mechano-perception plays a role in their responses to increased environmental salinity. Broadly speaking, an increase in environmental salinity can impact on plant cells in two ways: firstly, through osmotic reduction of cell turgor pressure, essentially a mechanical stimulus, and secondly, through the increased influx of Na$^{+}$, which may then compete with and displace K$^{+}$ from carboxylate groups of cell proteins such as actin. These impacts are coupled in salt-sensitive Chara cells. When turgor pressure is reduced in the presence of NaCl, the magnitude of Na$^{+}$ influx increases (Davenport, Reid & Smith 1996).

In this paper, we set out to compare and contrast the effects of turgor pressure reduction and elevated environmental salinity on mechano-perception in Chara cells. Because elevated extracellular Ca$^{2+}$ ion concentrations reduce the negative impact of high salinity in a wide range of plant cells (reviewed by Cramer 2002), including Chara (Hoffmann, Tufariello & Bisson 1989; Whittington & Smith 1992), we conducted our experiments in saline media including either high or low Ca$^{2+}$ concentrations. Elevated extracellular Ca$^{2+}$ diminishes both Na$^{+}$ influx via non-selective cation channels (NSCCs), and Na$^{+}$-induced K$^{+}$ efflux through outward rectifier channels in Arabidopsis (Shabala et al. 2006). We employed the powerful techniques of current–voltage (IV) analysis and mathematical modelling (Beilby & Walker 1996) to identify changes in ion transporters induced both by turgor pressure reduction and by saline media.

Plant physiological experiments frequently focus on a single environmental factor, such as salinity, or mechanical stimulus, when both factors are present in the natural world. Giant charophyte cells are unique experimental materials that enable investigation of the electrophysiological phenomena associated with mechano-perception under conditions of high salinity. Cells of embryophytes and charophytes respond to mechanical stimulus with a similar electrophysiological modus of receptor and action potentials (Shimmen 2001). Our results may have broad implications for understanding the interactions between mechano-perception and salinity stress in plant cells.

**MATERIALS AND METHODS**

**Cell preparation**

Chara corallina was cultured in Japan as described previously (Mimura & Shimmen 1994). Chara australis Brown (Garcia & Chivas 2006) was collected from Little Bay, Sydney, New South Wales, Australia, and was planted in aquaria containing a handful of autoclaved garden soil, a handful of rotting leaves and rainwater. C. australis was cultured under equal numbers of Sylvania Gro-Lux fluorescent tubes (Sylvania Australasia Pty. Ltd., Lisarow, New South Wales, Australia) and cool white fluorescent tubes, providing a photosynthetically active radiation of 80 μmol m$^{-2}$ s$^{-1}$, on a cycle of 10 h light and 14 h darkness. The C. corallina internodal cells used in mecano-stimulus experiments were between 45 and 53 mm long. Cells were cut from vigorous plants and were kept in artificial pond water (APW; Table 1a) in a growth cabinet for at least a week before experiments. Experiments were conducted at room temperature (28 °C).
The *C. australis* internodal cells used in voltage-clamp experiments were about 10–15 mm in length. Sub-apical internodal cells were cut from healthy plants and were left to recover in APW for at least 3 d. Experiments were conducted at room temperature (26 °C).

*C. australis* internodal cells were also used in cell viability tests.

### Experimental protocols

We used three experimental protocols: (1) mechanostimulation, (2) I/V measurements and (3) cell viability tests.

### Mechano-stimulation experiments

We measured and compared responses with mechanostimulus in the media listed in Table 1a.

Figure 1 shows the experimental protocols. Firstly, internodal cells were placed into a Perspex chamber (inhause), with two compartments (A and B) connected by a groove in the Perspex. The compartments A and B were electrically isolated using petroleum jelly. Both compartments initially contained APW, in which cells remained for at least 30 min.

Mechano-stimulus dislodges impaled microelectrodes and damages the cells, and we used extracellular electrodes to record difference potentials (Shimmen 1996). A difference potential is the difference in electrical potential between the two electrically isolated ends A and B of the cell. The membrane potential difference (PD) at each end of the cell is similar in APW, and hence $E_A - E_B$ is initially close to zero.

The electrical PD between compartments A and B ($E_A - E_B$) was measured using two 3 m KCl-agar electrodes ($E_A$ and $E_B$) connected to Ag/AgCl wire and inserted into compartments A and B, respectively. Signals from the electrodes were amplified using a differential microelectrode amplifier (MEZ7101; Nihon Kohden, Tokyo, Japan), and the signal $E_A - E_B$ (the difference potential) was recorded using a chart recorder (VP-6521A; National, Tokyo, Japan) with variable speed and sensitivity.

We used the K+ anesthesia technique to measure cell PDs (Shimmen, Kikuyama & Tazawa 1976). The 180sorb solution was introduced into both compartments to reduce turgor pressure (Fig. 1a). The 180sorb in compartment B was then exchanged for an isotonic solution of 100 KCl (Fig. 1b). Because the PD is close to zero in 100 KCl, $E_A - E_B$ gives the membrane PD of the part of the cell in compartment A.

Cells were mechanically stimulated using the device constructed by Shimmen (1996). Briefly, a small polyacrylate stimulator (dimensions, 5 × 8 × 1 mm) was suspended adjacent to the cell flank in compartment A. The stimulator was impacted by a thin glass rod (weight, 1.28 g) released from increasing heights (0.5–6 cm). The device delivers a series of reproducible mechanical stimuli (f0.5, f1, f2, f3 and f4) of increasing energy (see Shepherd et al. 2001). Stimulations were applied until an AP was induced. We avoided summation of the receptor potentials (Kishimoto 1968) by leaving 4–5 min between stimulations. Cells were allowed to recover for at least an hour following an AP.

In separate experiments, we mechanically stimulated cells in 100 Na/1.0 Ca and in 100 Na/0.1 Ca (Fig. 1c). The 180sorb in compartment B was exchanged for either 100 Na/1.0 Ca or 100 Na/0.1 Ca, and the cell was left for 60 min to stabilize Ca$^{2+}$/Na$^+$ exchange in the cell wall.

In one group of experiments, we compared mechanoreponses of the same cells in 100 Na/1.0 Ca and in 100 Na/0.1 Ca.

### Table 1. Chemical composition and acronyms for media used in (a) mechano-perception experiments, (b) voltage-clamp experiments and (c) cell viability tests

<table>
<thead>
<tr>
<th>Solution acronym</th>
<th>KCl (mM)</th>
<th>NaCl (mM)</th>
<th>CaCl$_2$ (mM)</th>
<th>Sorbitol (mM)</th>
</tr>
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<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APW</td>
<td>0.1</td>
<td>1.0</td>
<td>0.1</td>
<td>–</td>
</tr>
<tr>
<td>180sorb</td>
<td>0.1</td>
<td>1.0</td>
<td>0.1</td>
<td>180.0</td>
</tr>
<tr>
<td>100 KCl</td>
<td>100.0</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>100 Na/1.0 Ca</td>
<td>0.1</td>
<td>100.0</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>100 Na/0.1 Ca</td>
<td>0.1</td>
<td>100.0</td>
<td>0.1</td>
<td>–</td>
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<tr>
<td>(b)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APW</td>
<td>0.1</td>
<td>1.0</td>
<td>0.1</td>
<td>–</td>
</tr>
<tr>
<td>90sorb</td>
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<td>0.1</td>
<td>90.0</td>
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<tr>
<td>50 Na/1.0 Ca</td>
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<td>50.0</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>50 Na/0.1 Ca</td>
<td>0.1</td>
<td>50.0</td>
<td>0.1</td>
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<tr>
<td>(c)</td>
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<tr>
<td>APW</td>
<td>0.1</td>
<td>1.0</td>
<td>0.1</td>
<td>–</td>
</tr>
<tr>
<td>180sorb</td>
<td>0.1</td>
<td>1.0</td>
<td>0.1</td>
<td>180.0</td>
</tr>
<tr>
<td>100 Na/10 Ca</td>
<td>0.1</td>
<td>100.0</td>
<td>10.0</td>
<td>–</td>
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<tr>
<td>100 Na/1.0 Ca</td>
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<td>1.0</td>
<td>–</td>
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<tr>
<td>100 Na/0.1 Ca</td>
<td>0.1</td>
<td>100.0</td>
<td>0.1</td>
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</table>

The pH of all the solutions was 7.0, maintained by the addition of 5.0 mM HEPES-Tris. APW, artificial pond water; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Cells stimulated in 100 Na/1.0 Ca were left to recover for 60 min, and the 100 Na/1.0 Ca was exchanged for 100 Na/0.1 Ca. Cells were mechanically stimulated 30–45 min later (Fig. 1d).

We recorded the cell resting membrane PDs, the peak amplitudes of receptor potentials, the occurrence of touch-induced action potentials, and PD oscillations.

In a control experiment, we subjected a group of cells to the protocol of repetitive solution changes and mechanical stimulus, using only APW. As found previously (Shepherd et al. 2001), these procedures had only slight effect on the mechano-responses.

### The I/V measurements

Cells did not survive voltage-clamping protocols in 100 mM NaCl solutions, regardless of the Ca²⁺ concentration. We reduced the NaCl concentration to 50 mM, containing 0.1 mM Ca²⁺ or 1.0 mM Ca²⁺. This increased the activity of Ca²⁺ from 0.58 (in 100 mM NaCl) to 0.65 in 50 mM NaCl (Butler 1968). We reduced cell turgor pressure using isotonic 90sorb. Table 1b lists the chemical composition of the media.

The voltage-clamping procedure was as previously described (Beilby & Shepherd 1996). Cells were
compartment-clamped, with a microelectrode located in the cytoplasm or vacuole. The plasma membrane I/V characteristics dominate because of the high tonoplast conductance (Beilby 1990). We measured I/V characteristics using a bipolar staircase voltage command, with pulses of width between 60 and 100 ms, separated by 120–250 ms, at the resting PD.

The order and time course of solution changes mimicked those in mechano-stimulus experiments. We first obtained I/V characteristics of cells in APW and in 90sorb. We then measured I/V characteristics of cells transferred into either 50 Na/1.0 Ca or 50 Na/0.1 Ca. We also compared I/V characteristics of the same cells in 50 Na/1.0 Ca and 50 Na/0.1 Ca.

**Modelling and analysis of I/V data**

We resolved the total current into contributions made by parallel populations of ion transporters. We modelled the electrogenic proton pump at the plasma membrane using the two-state Hansen, Gradmann, Sanders and Slayman (HGSS) model (Hansen et al. 1981). We fitted the inward and outward rectifiers using the Goldmann, Hodgkin and Katz (GHK) model, supplemented by the Boltzmann distribution (Amtmann & Sanders 1999). We used an empirical model of the ‘background current’, with a reversal PD of −100 mV and a PD-independent conductance (Beilby & Shepherd 2006a; Al Khazaaly & Beilby 2007). Channels passing the ‘background current’ are the probable equivalent of NSCCs (non-selective cation permeable channels) found in land plants (Beilby & Shepherd 2001).

**Cell viability tests**

We assessed cell viability from the rate of cytoplasmic streaming in control cells, where mechano-stimulus was avoided. Cells cut from healthy plants were left in APW for 1 week. Ten cells were transferred into each of the media (Table 1c). The media were replaced every second day, and care was taken to avoid mechano-stimulus. We estimated the rate of cytoplasmic streaming as the time taken for cytoplasmic particles to traverse a 32¥ field of view. The number of viable cells was counted daily over 7 d. Dead cells were removed.

**RESULTS**

Figure 2 shows excerpts from chart records of cell PDs, and cell mechano-responses, in 180sorb, 100 Na/1.0 Ca and 100 Na/0.1 Ca. Table 1 shows the composition of the media and explains the acronyms used for each. Table 2 shows the mean resting membrane PDs of cells in these media. Table 3a,b summarizes mechano-responses of cells in all experiments.

**Mechano-responses in APW and 180sorb**

The hyperpolarized PDs of cells in both APW and 180sorb (Table 2) were characteristic of the ‘pump state’. Resting PDs in these media were not significantly different. As reported previously (Shepherd et al. 2001), cells were sensitized to mechano-stimulus when turgor pressure was reduced. In 180sorb, cells responded to given stimuli with larger amplitude receptor potentials than in APW. Stimuli between f1 and f4 (modal value f4) induced action potentials in APW. Stimuli between f0.5 and f3 (modal value f0.5) induced action potentials in 180sorb.

In both APW and 180sorb, the amplitude and duration of receptor potentials increased incrementally with increased stimulus energy, until an action potential was induced (Fig. 2a,b; Table 3a,b).

**Mechano-responses in 100 Na/1.0 Ca**

Cells began depolarizing immediately in isotonic 100 Na/1.0 Ca, reaching a mean PD of −123 ± 21 mV after 60 min (Tables 2 and 3a; Fig. 2c), which was significantly different to that in APW (P<0.00). Small magnitude PD oscillations observed in APW and 180sorb increased in amplitude and period in all cells (Fig. 2c). These oscillations are not clearly visible at the scale of reproduction of the figure. We will describe such oscillations in detail in a forthcoming paper.

Most cells (3, 4, 6 and 7 in Table 3a) became more sensitive to mechano-stimulus in 100 Na/1.0 Ca (Fig. 2c, Table 3a). Cells 1 and 5 did not decrease their sensitivity to stimulus. However, the limited resolution of the apparatus did not enable us to determine if the sensitivity of these cells had increased. Because 100 Na/1.0 Ca and 180sorb were isotonic media, the increased sensitivity of most cells results from increased NaCl concentration. Cell 2 alone decreased its sensitivity to mechano-stimulus.

Initially, all cells recovered the resting PD following the touch-induced action potential. However, all cells (with the exception of 7) then began to depolarize after 45–60 s (Fig. 2d). When the cell PD approached a mean value of −90 ± 23 mV (n = 6, limits: −60 to −113 mV), the cells entered a series of spontaneous repetitive action potentials (Fig. 2d). The duration and form of these action potentials was unique to each cell and repeated over a period of up to 60 min. At their conclusion, cells gradually hyperpolarized to a mean PD of −118.2 ± 9.5 mV (Table 2). This PD remained stable for a further 60 min. Cell 7 alone did not depolarize further from its post-action potential PD of −135 mV, neither did it begin repetitive firing.

**Mechano-responses of the same cells in 100 Na/0.1 Ca**

After 60 min in 100 Na/0.1 Ca, cells depolarized further to −82 ± 43 mV (Tables 2 and 3a; Fig. 2e). The amplitudes of small-scale resting PD oscillations increased to between 5 and 10 mV (Fig. 2e) with occasional spikes of approximately 25 mV (not shown). We distinguished three types of electrophysiological behaviour prior to mechano-stimulus (Table 3a): (1) cells 1 and 7 fired a single spontaneous action potential. Cell 1 subsequently remained much depolarized.

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Cell 7 recovered its PD for a few seconds, then depolarized to zero PD and ceased streaming, and died; (2) cells 2 and 3 spontaneously entered repetitive action potentials, and then depolarized significantly; and (3) cells 4, 5 and 6 retained a similar PD to that in 100 Na/1.0 Ca (Fig. 2e).

Initiation of spontaneous action potentials was a markedly different behaviour. Spontaneous action potentials had only followed touch-induced action potentials in 100 Na/1.0 Ca.

Responses to mechano-stimulus differed from those in 100 Na/1.0 Ca. Most cells (1, 2, 3 and 6 in Table 3a) decreased their sensitivity to mechano-stimulus and responded to given stimuli with smaller amplitude receptor potentials. Of these, only cells 2 and 6, with PDs of −81 and
Figure 2. Responses to mechanical stimulation of a Chara cell in 180sorb, 100 Na/1.0 Ca and 100 Na/0.1 Ca. The composition of the media is shown in Table 1. Results (a–f) are from the same cell throughout. Vertical bars indicate where the chart recorder speed was slowed, and the time scale is in minutes. (a) Cell difference potentials (see Fig. 1a), with turgor pressure reduced in 180sorb. The cell responds to increasing mechanical stimuli f1 and f2 with receptor potentials; small, transient depolarizations. Stimulus f3 induces an action potential (AP). The asterisk (*) symbol marks the threshold receptor potential that initiates the AP. The AP appears biphasic because a touch-induced AP occurred in both ends of the cell. Note that the difference potential does not necessarily show the true amplitude of the response. (b) Cell membrane potential differences (PDs), measured by K+ anesthesia, with turgor pressure reduced in 180sorb (see Fig. 1b). The resting membrane PD was −225 mV. As described previously, the cell responds to mechanical stimuli f1 and f2 with receptor potentials of increasing amplitude but similar duration (9 mV and 35 s for f1, and 18 mV and 35 s for f2), and stimulus f3 induces an AP. The touch-induced AP appears monophasic because cell part B (see Fig. 1b) has zero PD. The cell recovers its resting PD following the touch-induced AP. (c) Cell membrane PD in isotonic 100 Na/1.0 Ca. The y-axis is located 20 min after introducing 100 Na/1.0 Ca. The cell began depolarizing immediately (not shown), reaching a stable PD of −137 mV after 45 min. Small-scale PD oscillations (within vertical bars) were measured with the chart speed set to a time scale of minutes. These oscillations are not clearly visible at the scale of reproduction of the figure. We will describe them in detail in a forthcoming paper. Oscillation amplitudes were between 2 and 5 mV, and their duration, approximately 1 min. These amplitudes and periods were larger than small-scale oscillations in 180sorb (not shown). The cell increased its sensitivity to mechanical stimuli, in comparison with its responses in 180sorb. Stimulus f0.5 provoked a receptor potential (7 mV amplitude: 15 s duration). Small-scale PD oscillations are visible during the 4 min recovery period (vertical bars). Stimulus f1 provoked a receptor potential (15 mV amplitude: 60 s duration) and stimulus f2 induced an AP. (d) Following the touch-induced AP in isotonic 100 Na/1.0 Ca, the cell briefly recovered the resting PD of −137 mV, and then, 60 s after the touch-induced AP began to depolarize. The first panel of the chart excerpt shows this depolarization. At a PD of −70 mV, the cell entered a series of spontaneous repetitive APs (*). These were of short duration (approximately 6 s) and occurred at similar intervals (170.5, 170.5, 170.5, 162, and 195 s). Following repetitive firing, the cell gradually recovered a stable resting PD of −116 mV (not shown). The cell increased its sensitivity to mechano-stimulus, the form of the AP changed, and spontaneous repetitive APs followed the touch-induced AP. (e) Membrane PD of the same cell in isotonic 100 Na/0.1 Ca. The y-axis is situated 35 min after introducing 100 Na/0.1 Ca. This cell retained a resting PD between −116 and −113 mV, as described previously. However, small-scale PD oscillations (arrow) increased in amplitude (between 2 and 10 mV) and duration (approximately 2.5 min). Sensitivity to mechanical stimulus increased, and stimulus f0.5 provoked a receptor potential (12 mV amplitude: 30 s duration). Small-scale PD oscillations are visible (vertical bars). Stimulus f1 induced an AP of extended duration (3 min 30 s). (f) Repetitive APs followed the touch-induced AP. The cell initially recovered the resting PD, and then gradually depolarized (not shown), initiating repetitive APs at a PD of −26 mV. Compared with repetitive APs in 100 Na/1.0 Ca, these APs had extended duration (approximately 20 s) and occurred at shorter intervals (26, 30, 37, 36, 33, 33 and 34 s). The APs ceased when the cell PD hyperpolarized to −75 mV (not shown). Unusually, this cell recovered a stable PD of −107 mV. Following further repetitive APs, the cell died within 60 min of mechano-stimulation. (g) Cell PD and mechano-responses of another cell, this time transferred directly from 180sorb into 100 Na/0.1 Ca. The cell PD in 180sorb was −230 mV. The y-axis is situated 10 min after introducing 100 Na/0.1 Ca. After 10 min, the cell had depolarized to a PD of −107 mV. Repetitive APs (*) commenced after 20 min. The time scale of the chart is in minutes rather than seconds, and the firing is not resolved as described previously. The PD stabilized at −22 mV following spontaneous firing. The cell was desensitized to mechano-stimulus. Stimulus f1 provoked an extended depolarization, rather than a receptor potential. The cell subsequently depolarized to 0 mV, and did not recover cytoplasmic streaming after 40 min.

Table 2. Membrane PDs (mean±SD, n = number of cells) of cells used in mechano-stimulation experiments in APW, 180sorb, 100 Na/0.1 Ca and 100 Na/1.0 Ca

<table>
<thead>
<tr>
<th>APW</th>
<th>180sorb</th>
<th>100 Na/0.1 Ca</th>
<th>100 Na/1.0 Ca</th>
<th>**100 Na/0.1 Ca</th>
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<tbody>
<tr>
<td>PD (mV)</td>
<td>PD (mV)</td>
<td>PD (mV)</td>
<td>PD (mV)</td>
<td>PD (mV)</td>
</tr>
<tr>
<td>−228 ± 42 (n = 6)</td>
<td>(a): −212 ± 23 (n = 6)</td>
<td>Initial: −106 ± 15 (n = 6)</td>
<td>noRAP: −121 (one cell)</td>
<td>noRAP: −123 ± 21 (n = 7)</td>
</tr>
<tr>
<td></td>
<td>(b): −217 ± 16 (n = 7)</td>
<td>RAP: −53 ± 41 (n = 6)</td>
<td>Post-MS: 0 (n = 6)</td>
<td>Post-MS: −118 ± 10 (n = 7)</td>
</tr>
</tbody>
</table>

The column **100 Na/0.1 Ca shows the PD of cells pre-treated in 100 Na/1.0 Ca. The PD in 180sorb of cells transferred into 100 Na/0.1 Ca (a) and 100 Na/1.0 Ca (b) was similar. ‘Initial’ signifies the stable PD after 30 min in the solution. RAP signifies repetitive action potentials (shown in Figures 2d, f, g), and the accompanying value is the mean PD after they ceased. noRAP indicates the absence of repetitive action potentials. ‘Post-MS’ is the mean PD 60 min after mechano-stimulation in each solution. In column two, the two groups of cells (a) and (b) were subsequently transferred into 100 Na/0.1 Ca or 100 Na/1.0 Ca.

PDs, potential differences; APW, artificial pond water; MS, mechano-stimulus.

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Table 3. (a) Mechano-responses of Chara cells with turgor reduced in 180sorb in isotonic 100 Na/1.0 Ca and of the same cells in isotonic 100 Na/0.1 Ca. (b) Mechano-responses of Chara cells with turgor reduced in 180sorb and of the same cells in isotonic 100 Na/0.1 Ca

<table>
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<tr>
<th>Cell</th>
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<td>Receptor potential amplitude (mV) at stimulus</td>
<td>Stable PD (mV)</td>
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<tr>
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<td>f1: TAP</td>
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<td>Receptor potential amplitude (mV)</td>
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<td>3</td>
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<tr>
<td>4</td>
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<td>5</td>
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<td>f3: TAP</td>
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<tr>
<td></td>
<td></td>
<td>Stable PD: -205</td>
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</table>
and within 60 min (Fig. 2g). To zero PD, cessation of cytoplasmic streaming, and death. Mechano-stimulus was followed by gradual depolarization potentials or became entirely unresponsive (Fig. 2g). These produced smaller-amplitude receptor stimulation (Table 3b), became less sensitive to mechano-responses in 180sorb (Table 3b, Fig. 2g).

Cells responded to mechano-stimulus in 100 Na/0.1 Ca with a different spectrum of responses compared with their responses in 180sorb (Table 3b, Fig. 2g).

Cells 1, 3 and 5, which were much depolarized prior to stimulation (Table 3b), became less sensitive to mechano-stimulus. These produced smaller-amplitude receptor potentials or became entirely unresponsive (Fig. 2g). Mechano-stimulus was followed by gradual depolarization to zero PD, cessation of cytoplasmic streaming, and death within 60 min (Fig. 2g).

In contrast, cells 2 and 4 fired touch-induced action potentials (Table 3b). The action potentials were followed in both these cells by spontaneous repetitive firing, after which cells depolarized to zero PD, ceased streaming, and died.

Cell 6 alone became more sensitive to mechano-stimulus, responding to given stimuli with receptor potentials of larger amplitude and an action potential with a lower energy stimulus than in 180sorb (Table 3b). This cell also fired spontaneously and repetitively, depolarized, and died.

Thus, while all cells stimulated in 100 Na/1.0 Ca recovered a relatively hyperpolarized PD (−118.2 ± 9.5 mV) and survived for longer than 60 min post-stimulus, mechano-stimulus in 100 Na/0.1 Ca was quickly lethal.

Mechano-responses of cells transferred directly from 180sorb into 100 Na/0.1 Ca

All cells initially depolarized by approximately 100 mV to a mean PD of −106 ± 15.1 mV, after transfer from 180sorb to 100 Na/0.1 Ca (Fig. 2g; Tables 2 and 3b). As discussed previously, the amplitudes of small-scale resting PD oscillations also increased to between 5 and 10 mV with occasional spikes of approximately 25 mV amplitude. The cells were significantly depolarized (Pb = 0.00) in comparison with their PD in APW and 180sorb (Table 2), but only slightly depolarized in comparison with the PD of the first group of cells, in 100 Na/1.0 Ca (Pb = 0.19). However, after approximately 10–20 min in 100 Na/0.1 Ca, cells (except 2) fired spontaneous repetitive action potentials (Table 3b, Fig. 2g).

At the conclusion of repetitive firing, cells were further depolarized to a mean PD of −53 ± 41 mV (Table 2). Half the cells were much depolarized (with PDs close to −22 mV), and half had PDs ranging from −80 to −112 mV (Table 3b). Cells were significantly more depolarized in 100 Na/0.1 Ca than in 100 Na/1.0 Ca (Pb = 0.01) because of repetitive firing.

Cells responded to mechano-stimulus in 100 Na/0.1 Ca with a different spectrum of responses compared with their responses in 180sorb (Table 3b, Fig. 2g).

Cells 1, 3 and 5, which were much depolarized prior to stimulation (Table 3b), became less sensitive to mechano-stimulus. These produced smaller-amplitude receptor potentials or became entirely unresponsive (Fig. 2g). Mechano-stimulus was followed by gradual depolarization to zero PD, cessation of cytoplasmic streaming, and death within 60 min (Fig. 2g).

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Current–voltage analysis and mathematical modelling

Neither the resting PD nor the I/V characteristics changed significantly when turgor pressure was reduced in 90sorb (Fig. 3a–d, data in APW in black, data in 90sorb in blue).

The resting PD depolarized from −236 ± 7 to −184 ± 20 mV, and the conductance increased approximately threefold (Table 4; Fig. 3a–d, data in green) after 60 min in 50 Na/1.0 Ca. Modelling identified classes of transporters responsible. The background conductance increased from 0.45 ± 0.15 to 2.0 ± 0.3 S·m⁻². The rate constant parameters k₀ and kₐ of the electrogenic proton pump both increased from 8000 ± 2000 and 100 ± 20 s⁻¹ in APW to 12 000 ± 3000 and 130 ± 25 s⁻¹, respectively (parameter values in Table 4). This group of cells did not survive voltage clamping to potentials more negative than −280 mV, and so we could not investigate inward rectifiers.

The same cells, when transferred to 50 Na/0.1 Ca, depolarized further to −155 ± 7 mV. The PD range, where clamping was feasible, also narrowed (Fig. 3a–d, data in red). Only two of the group of five cells survived voltage clamping, and only for ~30 min. There was no further change in the background conductance. Both of the rate

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constant parameters $k^c_i$ and $k^i_i$ of the electrogenic proton pump decreased to $8000 \pm 2500$ and $60 \pm 25$ s$^{-1}$, respectively.

A separate group of four cells was transferred directly into 50 Na/0.1 Ca. Of these, only one showed a low level of proton pump activity (Table 4) after 60 min. The averaged data were modelled by the background conductance only (Fig. 3a-d in orange). The background conductance increased significantly to $3.2 \pm 0.2$ S·m$^{-2}$.

### Viability and rates of cytoplasmic streaming

Figure 4 shows the viability of control cells that were not mechanically stimulated in each of the media (Table 1c). Cell viability depended on both the external concentration of NaCl and Ca$^{2+}$, but not on the extracellular osmotic pressure: viability in 180sorb was slightly greater than in APW. All cells had died within 5 d in 100 Na/0.1 Ca, while half were still viable after 7 d in 100 Na/1.0 Ca. Viability depended on extracellular [Ca$^{2+}$]$_{ext}$: all cells survived for 7 d in modified APW including 10 mM Ca$^{2+}$ (not shown), whereas only 70% survived for 7 d in APW containing 0.1 mM Ca$^{2+}$. The rate of cytoplasmic streaming ($65 \pm 10$ μm s$^{-1}$) did not change significantly after 3 d in any medium except 100 Na/1.0 Ca and 100 Na/0.1 Ca, where it slowed to 50–45 μm s$^{-1}$, respectively. These unstimulated cells remained viable for significantly longer in saline media than their mechanically stimulated counterparts.

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DISCUSSION

Historically, the use of simpler cell models has facilitated understanding of complex multicellular systems, and giant charophyte cells are prototypes of plant cell excitability, membrane transport (Tazawa & Shimmen 2001) and mechano-perception (Shimmen 2001). We have investigated the effects of salinity on mechano-perception by Chara cells, as manifested in receptor and action potentials, as well as salinity-induced changes in electrophysiology. Reducing cell turgor pressure increases sensitivity to mechanical stimulus in Chara cells (Shepherd et al. 2001). Here, we report that an isotonic increase in salinity has effects on the receptor potential and excitability, over and above the effect of reduced turgor pressure. The nature of these effects depends on the extracellular Ca\(^{2+}\) ion concentration.

Salinity-induced changes in ion transport: the points of no return

The PD of plant cells contains contributions from two components: first, the passive (background conductance), and second, the active and pump-specific. Reducing turgor pressure had negligible effects on these components (Fig. 3, Table 4; Shepherd et al. 2001). Similarly, mannitol-induced turgor reduction has little effect on the proton pump activity in Mung bean (Nakamura et al. 1992). However, I/V

Table 4. Parameters used in mathematical modelling

<table>
<thead>
<tr>
<th>Medium</th>
<th>(g_{\text{background}}) (S m(^{-2}))</th>
<th>(k_{00}^\text{io}) (s(^{-1}))</th>
<th>(k_{00}^\text{oi}) (s(^{-1}))</th>
<th>Resting PD (mV)</th>
<th>Pump reversal PD (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APW</td>
<td>0.45 ± 0.15</td>
<td>8 000 ± 2000</td>
<td>100 ± 20</td>
<td>−236 ± 7</td>
<td>−377 ± 13</td>
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<td>9osorb</td>
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</tr>
<tr>
<td>Average time: 96 min</td>
<td>0.6 ± 0.3</td>
<td>8 800 ± 2800</td>
<td>100 ± 20</td>
<td>−227 ± 7</td>
<td>−379 ± 15</td>
</tr>
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<td>50 Na/1.0 Ca</td>
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<tr>
<td>Average time: 65 min</td>
<td>2.0 ± 0.3</td>
<td>12 000 ± 3000</td>
<td>130 ± 25</td>
<td>−184, range: -202, -167</td>
<td>−394 ± 12</td>
</tr>
<tr>
<td>50 Na/0.1 Ca after 50 Na/1.0 Ca</td>
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</tr>
<tr>
<td>Average time: 20–30 min</td>
<td>2.0 ± 0.3</td>
<td>8 000 ± 2500</td>
<td>60 ± 25</td>
<td>−150, range: -169, -127</td>
<td>−364 ± 23</td>
</tr>
<tr>
<td>50 Na/0.1 Ca after 9osorb</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average time: 60 min</td>
<td>3.2 ± 0.2</td>
<td>8 000 (max)</td>
<td>60 (max)</td>
<td>−100, range: -132, -60</td>
<td>−364 (max)</td>
</tr>
</tbody>
</table>

The other pump parameters, \(k_{00}^\text{io}\) and \(k_{00}^\text{oi}\), were kept at 0.5 s\(^{-1}\). The inward rectifier was not observed in the data sets. The uncertainties in the resting PD were estimated from the measured resting PDs in APW and 9osorb. In the 50 Na data sets, the range is defined by an en dash. The outward rectifier was observed only in the last set of data (bottom row): \(N_{\text{K}}F_{\text{pK}} = 0.65 \times 10^{-7}\) m s\(^{-1}\), \(V_{\text{K}} = -5\) mV, \(z_{g} = 2.0\). The curve of the best fit contained no contribution from the pump, while the upper limit contained the same contribution from the pump as in the previous data set. The lower limit contained no contribution from the pump, and the reversal PD for the background current changed to −60 mV. Cells were in the various media for the following average times when sampling was performed: 9osorb, 96 min; 50 Na/1.0 Ca, 65 min; 50 Na/0.1 Ca, after 50 Na/1.0 Ca, 20–30 min; and 50 Na/0.1 Ca after 9osorb, 60 min.

PD, potential difference; APW, artificial pond water.

![Figure 4](#)

**Figure 4.** Viability of cells, assessed by cytoplasmic streaming, in the absence of mechano-stimulus. Cells were in the saline and sorbitol-based media shown in Table 1c. Artificial pond water (APW) containing 10.0 mM Ca\(^{2+}\) was also tested, and all cells survived for 7 d.
analysis reveals four salinity-specific changes in ion transport, the nature of which depends on [Ca\textsuperscript{2+}]\textsubscript{ext}.

Firstly, a rapid and dramatic increase in the background conductance partially short-circuits the proton pump current, and thus depolarizes the membrane PD (Fig. 3, Table 4). The background conductance increased sevenfold in 50 Na/0.1 Ca [with low (Ca\textsuperscript{2+})\textsubscript{ext}] and fourfold in 50 Na/1.0 Ca [with high (Ca\textsuperscript{2+})\textsubscript{ext}]. If the background conductance in charophyte cells arises from the equivalent of NSCCs (Beilby & Shepherd 2001), it is also a likely route for Na\textsuperscript{+} influx, which occurs via NSCCs in embryophyte plant cells (Demidchik & Maathuis 2007). Calcium ions also inhibit NSCCs of Arabidopsis protoplasts in a concentration-dependent manner (Demidchik & Tester 2002).

Secondly, currents generated by the electrogenic proton pump changed in a calcium-dependent manner, increasing in 50 Na/1.0 Ca and declining to negligible levels in 50 Na/0.1 Ca (Fig. 3b, Table 4). Activation of the proton pump appears to depend on [Ca\textsuperscript{2+}]\textsubscript{ext}, or specifically on the ionic ratios between [Na\textsuperscript{+}]\textsubscript{ext} and [Ca\textsuperscript{2+}]\textsubscript{ext} at constant [K\textsuperscript{+}]\textsubscript{ext}. Chara cells depolarize to the passive component of the PD in Ca\textsuperscript{2+}-free APW (Bisson 1984). Stimulation of proton pumping in 50 Na/1.0 Ca did not counteract salinity-induced depolarization of the resting PD (Fig. 3a), but established a PD (Table 4) negative to the excitation threshold (AP\textsubscript{threshold}) of approximately −100 mV (Beilby 1990). Stimulation of proton pumping in saline media was described in Mung bean (Nakamura et al. 1992) and bean mesophyll (Shabala 2000). With negligible proton pumping (Fig. 3b), the background conductance becomes dominant in 50 Na/0.1 Ca, shifting the resting PD to AP\textsubscript{threshold} (Table 4, bottom row).

Thirdly, most cells in 100 Na/0.1 Ca spontaneously initiated trains of repetitive firing (Table 3a,b; Fig. 2g). This repetitive firing resembles the onset of a critical instability, of the type described by Hayashi & Hirakawa (1980) in the Nitella cell, where a time-ordered structure (repetitive firing) emerged after ramping currents to the excitation threshold. Most cells in 100 Na/1.0 Ca had PDs negative to AP\textsubscript{threshold} (Table 2), and initiated repetitive firing only after a touch-induced action potential, which shifted the PD transiently to AP\textsubscript{threshold} (Fig. 2d). Kishimoto (1966) attributed NaCl-induced repetitive firing in Nitella to calcium depletion at the cell membrane. Our results suggest this is because proton pump currents decline when [Ca\textsuperscript{2+}]\textsubscript{ext} is low, shifting the resting PD to AP\textsubscript{threshold}.

Finally, the threshold for activation of the outward rectifiers (KOR\textsubscript{threshold}) is approximately −50 mV (Fig. 3d), and depolarization to more positive potentials leads to sustained K\textsuperscript{+} efflux. When [Ca\textsuperscript{2+}]\textsubscript{ext} is low, increased background conductance and loss of proton pump activity combine to gradually depolarize the cell PD. Trains of spontaneous action potentials, which involve net Cl\textsuperscript{−} and K\textsuperscript{+} effluxes (Beilby 2001), accelerate the depolarization because the deactivation of the proton pump precludes recovery of the resting PD.

Thus, two critical thresholds, AP\textsubscript{threshold} and KOR\textsubscript{threshold}, serve as ‘points of no return’ when [Ca\textsuperscript{2+}]\textsubscript{ext} is low. Proton pump inactivation depolarizes cells to AP\textsubscript{threshold}, initiating spontaneous repetitive firing and further depolarization to KOR\textsubscript{threshold}. At this point, K\textsuperscript{+} depletion becomes irreversible. The background conductance increases to a lesser extent when [Ca\textsuperscript{2+}]\textsubscript{ext} is high, and increased proton pump activity establishes a PD outside the danger zone of the critical AP\textsubscript{threshold}. In the absence of mechano-stimulus, this circumvents both ‘points of no return’.

Interestingly, cells pre-treated in saline media with high [Ca\textsuperscript{2+}]\textsubscript{ext} had more negative PDs than those transferred directly to media with low [Ca\textsuperscript{2+}]\textsubscript{ext} (Tables 3a and 4). Such pre-treatment may delay the eventual displacement of Ca\textsuperscript{2+} by Na\textsuperscript{+} at negative sites in the cell wall (Shabala & Newman 2000).

We do not yet know whether excitation plays a role in responses of land plant cells to salinity. In the 1990s, whole-cell patch clamp studies of various cereal roots suggested the possibility that the K\textsuperscript{−} outward rectifier could maintain an outward K\textsuperscript{−} current while less selective cation channels permit Na\textsuperscript{+} entry (reviewed by Tyerman & Skerrett 1999). Salinity induces K\textsuperscript{+} efflux via the outward rectifier in land plant cells (Shabala et al. 2006), and this depends on the magnitude of NaCl-induced depolarization (Chen et al. 2007), or, essentially, on proton pump activity, as in Chara. Land plants are a sister group to the extant Charales (Karol et al. 2001), and these cellular responses to salinity are likely to be fundamental, with ancient antecedents.

The salt-tolerant turgor-regulating charophytes, such as Chara longifolia (Yao, Bisson & Brzezicki 1992) and Lamprothamnium succinctum (Beilby & Shepherd 2001), avoid the ‘points of no return’ through activation of the proton pump, which maintains a negative resting PD (reviewed by Beilby, Bisson & Shepherd 2006). Decreased turgor pressure activates the proton pump in Lamprothamnium (Al Khazaaly & Beilby 2007). Similarly, Thellungiella, a salt-tolerant relative of Arabidopsis, maintains a negative resting PD and regulates turgor during salt stress (Inan et al. 2004; Volkov & Amtmann 2006).

Mechano-perception in saline media as a function of [Ca\textsuperscript{2+}]\textsubscript{ext}

The receptor potential difference (RPD) involves activation of mechanosensitive Ca\textsuperscript{2+} channels at the plasma membrane, transient elevation of intracellular Ca\textsuperscript{2+} and efflux of Cl\textsuperscript{−} via Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels, resulting in transient depolarization (Kaneko et al. 2005). The magnitude of Ca\textsuperscript{2+} influx increases with increasing stimulus strength, accounting for incremental depolarization (RPD amplitude).

Most cells became hypersensitive to mechanical stimulus in 100 Na/1.0 Ca. The RPD amplitudes increased, relative to RPD amplitudes in sorbitol (Fig. 2a–c, Table 3a). This was an effect of NaCl, not simply a result of turgor reduction. Cells were thereby more likely to fire a touch-induced action potential (Table 3a, Fig. 2c), followed by repetitive firing as the repolarizing PD (~90 ± 23 mV) approached AP\textsubscript{threshold} (Fig. 2d).
Stimulation of proton pumping enabled restoration of the resting PD. However, each action potential depletes the cell of $K^+$ and $Cl^-$ (Beilby 2007), transiently inhibits photosynthesis (Bulychev et al. 2004; Krupenina & Bulychev 2007), and presumably has further indirect impacts on electrogenic pump-driven transport via ATP depletion. Although cells re-established a PD negative to $AP_{\text{threshold}}$ and all remained viable for at least 60 min following mechano-stimulus, even slight mechano-stimulus produces multiple excitations and $K^+$ efflux. Hypersensitivity to mechano-stimulus thus increases the propensity for depolarization to $AP_{\text{threshold}}$ and $KOR_{\text{threshold}}$.

Cells in 100 Na/0.1 Ca responded to given stimuli more haphazardly. Following spontaneous repetitive firing, many depolarized cells (e.g. with a PD of $-22$ mV) were desensitized to mechano-stimulus, producing smaller-amplitude RPDs or becoming inexcitable (Table 3a,b; Fig. 2g). In contrast, cells maintaining a more negative PD (e.g. $-80$ to $-121$ mV) retained their sensitivity to mechano-stimulus. Some cells became hypersensitive, producing RPDs of larger amplitude and touch-induced action potentials (Fig. 2e). Following repetitive firing (Fig. 2f), the cells depolarized to a PD more positive than $KOR_{\text{threshold}}$ (Table 3a,b). Unsurprisingly, all cells died within 60 min of mechano-stimulus, whereas 50% of non-stimulated cells remained viable after 2 d in 100 Na/0.1 Ca, and 90% were viable after 2 d in 100 Na/1.0 Ca (Fig. 4). A high $[Ca^{2+}]_{\text{ext}}$ to $[Na^+]_{\text{ext}}$ ratio in the medium has previously been found to enhance the survival of $C. corallina$ cells in saline media (Tufariello, Hoffmann & Bisson 1988).

Furthermore, the ratio between $[Ca^{2+}]_{\text{ext}}$ and $[Na^+]_{\text{ext}}$ has dramatic effects on action potential duration. The duration of both touch-induced and spontaneous repetitive action potentials was shorter in 100 Na/1.0 Ca than in 100 Na/0.1 Ca (compare Fig. 2c,d with Fig. 2e,f). Presumably, the excitation-associated net effluxes of $Cl^-$ and $K^+$ were greater in 100 Na/0.1 Ca. Monovalent cations, including Na+, prolong the action potential, but divalent ions, including Ca$^{2+}$, suppress this effect (Shimmen et al. 1976).

Increased salinity eventually induces fatal depolarization, depending on the $[Na^+]_{\text{ext}}$ to $[Ca^{2+}]_{\text{ext}}$ ratio (Fig. 4), but mechano-stimulus accelerates the process in a Ca$^{2+}$-dependent manner. A key factor in the fatal synergy between mechano-stimulus, $[Ca^{2+}]_{\text{ext}}$ and $[Na^+]_{\text{ext}}$ is excitation, both mechanically and spontaneously induced. Post-excitation recovery depends on proton pump activity re-establishing a PD negative to $AP_{\text{threshold}}$ and the pump is active only when $[Ca^{2+}]_{\text{ext}}$ is high. When $[Ca^{2+}]_{\text{ext}}$ is low, cells depolarize to $KOR_{\text{threshold}}$ following excitation.

**Cells are either desensitized or hypersensitized to mechano-stimulus in saline media: the roles of turgor pressure and $Cl^-$ conductance**

Superficially, the production of larger-amplitude RPDs (increased Ca$^{2+}$ influx and $Cl^-$ efflux) in saline media depends on membrane PD (Table 3a,b), but the generation of RPD does not depend on the $H^+$ pump (Shimmen 1997b). Two different factors contribute to the increased RPD amplitudes. The first lacks measurable electrophysiological correlates. As reported previously, cells are sensitized to mechano-stimulus when turgor pressure is reduced (Shepherd et al. 2001), but neither the cell PD nor the background conductance changes significantly (Fig. 3) (Kiyosawa & Ogata 1987; Shepherd et al. 2001).

We do not know why cells with reduced turgor are sensitized to mechanical stimulation. Decompression stimulus induces smaller-amplitude RPDs when turgor pressure is reduced, suggesting that changed membrane tension is a crucial factor (Iwabuchi et al. 2008). Changed membrane tension is unlikely to account for increased RPD amplitudes in our experiments, however, because membrane transfer between plasma membrane and endocytotic vesicles compensates for osmotic contraction in hypertonic solution (Wolfe & Steponkus 1983; Wolfe, Dowgert & Steponkus 1985; Dowgert, Wolfe & Steponkus 1987). The plasma membrane is probably under comparable small tension in both APW and 180osorb.

Previously, we suggested that a continuum among cell wall, plasma membrane and cytoskeleton (Baluska et al. 2003; Telewski 2006; Pickard 2007) is critical to mechano-perception in $Chara$ (Shepherd et al. 2001). Future experiments will investigate the effects on generation of receptor and action potentials of inhibiting either or both actin and microtubule cytoskeletons.

A second, electrophysiological factor contributes to increased RPD amplitudes. The hundredfold increase in $[Cl^-]_{\text{ext}}$ results in a reversal PD for $Cl^-$ ($E_C$) of approximately $-50$ mV, assuming a $[Cl^-]_{\text{cyt}}$ of 10 mM (Coster 1966). Thus, the driving force for $Cl^-$ efflux, on which the RPD depolarization depends, is negligible in cells with a resting PD close to $E_C$. At PDs more positive than $E_C$, the currents are diminished by the inwardly rectifying properties of the $Cl^-$ channels (Beilby & Shepherd 2006b). This is borne out by the data, where cells with PDs close to $E_C$ became unresponsive or showed reduced RPD amplitudes (Table 3a,b).

On the other hand, cells with more negative PDs were hypersensitized to mechano-stimulus, showing increased RPD amplitudes with given stimuli (Table 3a,b). Preliminary modelling, based on Beilby & Shepherd (2006b), shows that an increase in $[Cl^-]_{\text{cyt}}$ would result in an increase in $Cl^-$ currents, which may contribute to the observed hypersensitivity.

**Potential role of excitation in responses of land plants to salinity**

The action potentials of $Chara$ cells reflect those of land plant cells (Tazawa & Shimmen 2001; Fisahn et al. 2004), and mechanically stimulated or spontaneous excitations may thus be widely relevant to understanding land plant responses to salinity. Action and/or variation potentials suppress photosynthesis in $Chara$ (Krupenina & Bulychev 2007) and in land plants (Koziolek et al. 2004; Lautner et al. 2005). Salt (KCl) applied to roots induces action potentials in leaves (Favre, Greppin & Degli Agosti 2001; Felle &
Zimmermann 2007). Salinity reduces photosynthetic efficiency (e.g. Shabala et al. 2005), and mechano-perception and/or excitation may contribute. Excitation combined with proton pump inhibition is another possible avenue for the K⁺ depletion, which is thought (Maathuis & Amtmann 1999) to underlie NaCl toxicity. Circumvention of the critical $AP_{\text{threshold}}$ when $[\text{Ca}^{2+}]_{\text{ext}}/[\text{Na}^{+}]_{\text{ext}}$ is high potentially contributes to well-known ameliorative effects of high $[\text{Ca}^{2+}]_{\text{ext}}$ on salt-stressed plants (Cramer 2002).

**CONCLUSION**

Our results reveal the hitherto unrecognized importance of mechanically stimulated and spontaneous action potentials

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**Figure 5.** Diagrammatic representation of responses to mechano-stimulus in saline media with either high (1.0 mM) or low (0.1 mM) $\text{Ca}^{2+}$ ion concentrations. When $[\text{Ca}^{2+}]_{\text{ext}}$ is low, deactivation of the proton pump and the increase in background conductance depolarize the membrane potential to action potential ($AP_{\text{threshold}}$). Spontaneous repetitive excitations of extended duration are the result, followed by repolarization to potential differences (PDs) positive to $KOR_{\text{threshold}}$. Although these cells are desensitized to mechano-stimulus, K⁺ efflux is then sustained and irrevocable. On the other hand, cells retaining a PD negative to $KOR_{\text{threshold}}$ (and $E_{\text{Cl}}$) become hypersensitive to mechano-stimulus, and further excitation and K⁺ efflux then drive the PD to $KOR_{\text{threshold}}$. Cell viability was extended in the absence of mechano-stimulus. When $[\text{Ca}^{2+}]_{\text{ext}}$ is high, the smaller background current and stimulation of proton pump currents establish membrane PDs negative to $AP_{\text{threshold}}$. Cells do not spontaneously fire, but they become hypersensitive to mechano-stimulus. Even a small mechano-stimulus provokes excitation, followed by repetitive firing as the repolarizing PD crosses $AP_{\text{threshold}}$. $\text{Ca}^{2+}$-dependent activation of the proton pump re-establishes a PD slightly negative to $AP_{\text{threshold}}$ and well negative to $KOR_{\text{threshold}}$, and cells remain viable for longer when $[\text{Ca}^{2+}]_{\text{ext}}$ is high. Cell viability was extended in the absence of mechano-stimulus.
in the salt sensitivity of Chara cells. The cells eventually depolarize and die in saline media, but mechanically stimulated and spontaneous excitation augments the process in a Ca\textsuperscript{2+}-dependent manner. Two critical membrane potential thresholds, the excitation threshold (AP\textsubscript{threshold}) and the threshold for activation of the K\textsuperscript{+} outward rectifier (KOR\textsubscript{threshold}), act as ‘points of no return’ in determining cell viability in saline media. This is summarized in Fig. 5.

Molecular approaches have demonstrated the importance of calcium signalling in salt sensitivity (Zhu 2001). Action potentials function at a higher organizational level as systemic Ca\textsuperscript{2+} signals, and their role in the responses of land plants to increased salinity is an avenue for further study.

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Mechano-perception in Chara cells 1591

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