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Swelling-activated chloride channels in leech Retzius neurons

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SUMMARY

During periods of high activity neurons are expected to swell due to the uptake of Cl^-. To find out whether leech Retzius neurons possess swelling-activated Cl^- channels that facilitate Cl^- efflux and, hence, volume recovery, we exposed the cells to hypotonic solutions. In hypotonic solutions, the cells slowly swelled but did not undergo a regulatory volume decrease. However, the cell volume increased less than predicted for an ideal osmometer, suggesting the action of a compensatory mechanism. The cell swelling was paralleled by a marked decrease in the input resistance as well as by the activation of a membrane current with a reversal potential close to the Cl^- equilibrium potential. This current was substantially diminished by removing bath Cl^-, by applying the Cl^- channel blocker DIDS, or by treating the cells with the tubulin polymerization inhibitor colchicine. Furthermore, in the presence of colchicine or vinblastine, the cell swelling was substantially increased. It is concluded that leech Retzius neurons possess swelling-activated Cl^- channels that require an intact microtubule system for activation. The channels may help to restore cell volume after periods of high neuronal activity.

Key words: *Hirudo medicinalis*, osmotic shock, Retzius neuron, chloride channel, volume regulation.

INTRODUCTION

Animal cells are limited by a water-permeable membrane that cannot sustain hydrostatic pressure (Lang et al., 1998; Macknight et al., 1994). Consequently, the osmotic balance between the cytosol and the extracellular space determines the cell's water content and, hence, its volume. The osmolality of the extracellular solution is predominantly determined by dissolved inorganic ions, while that of the cytosol is to a considerable degree based on organic molecules, such as carbohydrates, amino acids and ATP. To create 'osmotic room' for these essential, mostly negatively charged molecules, Cl^- is kept out of the cytosol by the negative membrane potential (Armstrong, 2003). The fact that the membrane potential is ultimately based on the activity of the Na^+–K^+ pump implies that the maintenance of cell volume is an energy-consuming process.

Many observations have shown that the volume of a given cell is an important factor that affects a variety of cellular features, such as shape, metabolism and membrane transport (Wehner et al., 2003). Changes in cell volume can have many causes. Among them are the synthesis or degradation of macromolecules, the transport of osmolytes into or out of the cell, pathophysiological conditions such as insufficient water uptake by the organism, renal dysfunction or a disturbed energy supply due to hypoxia or ischaemia. Besides altering the concentrations of all cytosolic components, a change in cell volume may affect the interactions between cytosolic proteins ('molecular crowding'), the activity of proteins imbedded in the cell membrane (Janmey and Kinnunen, 2006), as well as the osmotic balance between the cytosol and the lumen of intracellular organelles.

Most cells have volume-regulating mechanisms that are activated when the cell volume deviates from its normal value, and which change the cytosolic osmolality such that the cell volume recovers more or less completely (Fürst et al., 2002; Lang et al., 1998; Macknight et al., 1994; O'Neill, 1999). Thus, cell swelling is usually followed by a regulatory volume decrease (RVD) and cell shrinkage by a regulatory volume increase (RVI). A rapid mechanism to counteract volume changes is the transport of organic and/or inorganic osmolytes across the cell membrane, either by membrane transporters or by membrane channels (Ellory and Hall, 1988; Okada, 1997). For an osmotically effective ion flux through ion channels, both cations and anions have to move across the cell membrane in order to satisfy the law of electroneutrality, which implies that the electromotive forces are directed such that the involved cations and anions flow in the same direction.

RVI is often mediated by an uptake of NaCl and RVD by a release of KCl. In order to drive Cl^- into or out of the cell, the electromotive force for Cl^- must be shifted correspondingly, by changing either the equilibrium potential for Cl^- or the membrane potential. In general, the Cl^- equilibrium potential is close to the resting membrane potential, so that the electromotive force for Cl^- is small. Therefore, to transport significant amounts of Cl^- across the cell membrane, the Cl^- conductance of the membrane must be high, which explains why many cell types express volume-sensitive, mostly swelling-activated Cl^- channels (Fürst et al., 2002). Swelling-activated Cl^- channels typically show slight outward rectification, have unitary conductances between 10 and 100 pS, and can be blocked by stilbene derivatives (Okada, 1997).

In neurons, cell swelling occurs not only under pathophysiological conditions, such as stroke or brain trauma, but also as a consequence of normal neuronal activity, such as synaptic transmission or the generation of action potentials (Andrew and MacVicar, 1994; Darquié et al., 2001; Rothman, 1985). In general, the excitability of swollen neurons is increased (Azouz et al., 1997; Müller et al., 2002) and, therefore, volume regulation seems to be essential for normal neuronal function. In this article, we show that

leech Retzius neurons respond to a reduction of the extracellular osmolality with a cell swelling that is paralleled by the activation of a membrane current. The properties of this current suggest that leech Retzius neurons possess swelling-activated Cl^- channels with an unusual pharmacology and a particular activation mechanism, which may augment the release of Cl^- after periods of high neuronal activity and hence accelerate the recovery of cell volume.

MATERIALS AND METHODS

Preparation

Leeches [*Hirudo medicinalis* L. (Annelida, Hirudinea)] were purchased from a commercial supplier (Zaug GmbH, Germany) or were taken from the lab's own breeding stock. The experiments were performed on Retzius neurons in intact segmental ganglia, which were dissected from the ventral nerve cord of adult leeches as previously described (Schlue and Deitmer, 1980). The ganglia were fixed ventral side up in an experimental chamber by piercing the connectives with fine steel needles and were continuously superfused with experimental solutions at a rate of 4 ml min^{-1}, which exchanged the chamber volume about 80 times per minute in the electrophysiological experiments, and 15 times per minute in the microfluorimetric measurements. All segmental ganglia were used for the experiments except those in segments 5 and 6, which show structural and functional peculiarities (Macagno, 1980). Before use, the ganglia were stored in a refrigerator (~8°C) for up to 8 h. The experiments were conducted at room temperature (~21°C).

Microfluorometry

In order to determine changes in cell volume *via* fluorescence emission, the cells were loaded iontophoretically with the fluorescent dyes Fura-2, BCECF or SBFI (Molecular Probes, Eugene, OR, USA), which are normally used to measure the cytosolic concentrations of Ca^{2+} ($[Ca^{2+}]_i$), H^+ ($[H^+]_i$) and Na^+ ($[Na^+]_i$) (see Haugland, 2002). For application as volume markers, the dyes were excited at their isosbestic points, at which the dye fluorescence is independent of the respective ion concentration, so that changes in the fluorescence emission reflect changes in the dye concentration and hence in cytosolic volume (Crowe et al., 1995; Gray et al., 1983; Muallem et al., 1992). For Fura-2, the isosbestic point is 360 nm, for BCECF it is 440 nm and for SBFI it is 370 nm. The following relationship between fluorescence emission and cytosolic volume was used (Alvarez-Leefmans et al., 1995):

$$\frac{F_0 - F_{BF}}{F_{exp} - F_{BF}} = \frac{V_{exp}}{V_0},$$

where F_0 and F_{exp} are the fluorescence of the cell under control and anisotonic conditions; F_{BF} is the background fluorescence without dye injection; and V_0 and V_{exp} are the cell volume under control and anisotonic conditions.

Besides monitoring cell volume, Fura-2, BCECF and SBFI were also used to measure the respective cytosolic ion concentrations by applying the ratio method (Grynkiewicz et al., 1985). In this method the indicator dye is excited alternately with two different wavelengths, and the ion concentration is determined from the ratio (R) of the fluorescence (F) excited by these wavelengths. In the case of Fura-2, excitation was at 340 nm and 380 nm, and $[Ca^{2+}]_i$ was calculated from $R=F_{340}/F_{380}$ (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d \left(\frac{R - R_{min}}{R_{max} - R}\right)\left(\frac{F_{f,380}}{F_{b,380}}\right),$$

where R_{min} is the minimum R measured in the absence of Ca^{2+}, and R_{max} the maximum R at saturating Ca^{2+} concentrations. $F_{f,380}/F_{b,380}$ is the fluorescence ratio of the Ca^{2+}-free and Ca^{2+}-saturated forms of Fura-2 upon 380 nm excitation; and K_d is the apparent dissociation constant of the Ca^{2+}–Fura-2 complex. The parameters R_{min}, R_{max} and $F_{f,380}/F_{b,380}$ were determined *in vitro* using solutions of Fura-2 in 100 mmol l^{-1} KCl, buffered to pH 7.4 with 50 mmol l^{-1} Hepes (Sigma, Taufkirchen, Germany) to which either 10 mmol l^{-1} $CaCl_2$ or 10 mmol l^{-1} of the calcium chelator EGTA (Sigma) were added. For K_d, a value of 135 nmol l^{-1} was used, which was measured under similar conditions (Grynkiewicz et al., 1985).

$[H^+]_i$ (or pH_i) and $[Na^+]_i$ were determined in an analogous way to $[Ca^{2+}]_i$. For BCECF the excitation wavelengths were 440 nm and 470 nm (James-Kracke, 1992), and the dye fluorescence was calibrated using solutions with different pH. For SBFI the excitation wavelengths were 340 nm and 380 nm. In contrast to Fura-2 and BCECF, the intracellularly recorded excitation spectrum of SBFI was shifted to longer wavelengths compared with excitation spectra recorded in aqueous solutions with different Na^+ concentrations. Therefore, an *in vitro* calibration of the SBFI fluorescence was not feasible, and the SBFI signal was calibrated by relating its fluorescence to $[Na^+]_i$ data obtained with Na^+-sensitive microelectrodes under identical conditions (Dierkes et al., 1996; Nett and Deitmer, 1998).

The experimental setup has been described previously in detail (Hochstrate and Schlue, 1995). Briefly, Retzius neurons were iontophoretically loaded with Fura-2, BCECF or SBFI (Molecular Probes) using single-barrelled microelectrodes, filled at their tip with the respective dye dissolved in water (30–100 mmol l^{-1}, electrode resistance 50–130 MΩ, injection current –10 nA for 60 s; iontophoretic amplifier: L/M-1, List, Darmstadt, Germany). About 2 min after dye injection the preparation was transferred into a flow chamber mounted on the stage of an inverted microscope (Diaphot-TMD, Nikon, Düsseldorf, Germany), which was part of a commercial microspectrofluorometer (Deltascan 4000, Photon Technology International, Wedel, Germany). The excitation light (three wavelengths applied alternately) was guided to the preparation *via* a ×40 objective (Fluor 40 Ph3DL, Nikon). The emitted fluorescence was collected by the same objective, passed through a barrier filter (BA 520/580, Nikon) to filter out the excitation light, and detected by a photon-counting photomultiplier tube with an acquisition rate of 1 s^{-1}. The measured object area was limited to a rectangular field by means of a variable diaphragm (15–20 μm edge length), which covered 5–10% of the cell's cross-section.

Confocal laser-scanning microscopy

We used a commercial confocal laser-scanning microscope (LSM, Leica TCS NT, Leica, Wetzlar, Germany) to visualize cell swelling and shrinkage in three dimensional reconstructions. Cells were iontophoretically loaded with the fluorescent dye Oregon Green BAPTA-1 (Molecular Probes). An Argon laser (488 nm) was used for fluorescence excitation, and the emitted fluorescence was separated from the excitation light by a band-pass filter (BP 525/50).

Electrophysiology

The soma of a Retzius neuron was impaled by two conventional electrolyte-filled microelectrodes, one for recording the membrane potential (E_m) and the other for current injection. The electrodes were pulled from borosilicate capillaries (outer/inner diameter: 1.5 mm/0.86 mm, 0.15 mm filament; Harvard Apparatus Ltd, Clark

capillaries, Edenbridge, UK) and filled with 0.5 mol l^{-1} K_2SO_4 and 5 mmol l^{-1} KCl. The bath electrode was an agar bridge containing 3 mol l^{-1} KCl and a chlorinated silver wire. The input resistance (R_{in}) of the cells was calculated from the hyperpolarization induced by injecting negative current pulses (–5 nA, duration 1 s, pulse interval 10 s), which were produced by a pulse generator (MAX 21, Zeitz-Instruments, Augsburg, Germany). The current-induced hyperpolarization usually showed a 'depolarizing sag' due to the activation of hyperpolarization-activated cation channels [I_h channels (Angstadt, 1999)]; i.e. after reaching an initial maximum, the hyperpolarization slightly declined to a stable plateau within a few hundred milliseconds (see Fig. 3B). For the calculation of R_{in}, the hyperpolarization was measured at the plateau, i.e. after full activation of the I_h channels. Membrane currents were measured at holding potentials (E_h) between –120 and –40 mV in 10 mV increments, with E_m being clamped for 1 s to the respective E_h in each step. In between steps (5 s), the cells were clamped to –50 mV. The measurements were performed using a two-electrode voltage-clamp amplifier (TEC-05L, NPI Instruments, Tamm, Germany) in the current-clamp or voltage-clamp mode. The output signals were digitized by an A/D converter (Digidata 1322A, Axon Instruments/Molecular Devices Corporation, Sunnyvale, CA, USA) and stored on an IBM-compatible PC. In current-clamp experiments the data acquisition rate was 100 or 200 Hz, and in voltage-clamp experiments it was 10 kHz.

Ion-sensitive microelectrodes

Experiments with the volume marker tetramethylammonium (TMA^+) were performed as described previously (Dierkes et al., 2002). Briefly, double-barrelled ion-sensitive microelectrodes were filled with the classical K^+ exchanger Corning 477317, and back-filled with 100 mmol l^{-1} KCl. The reference barrel was filled with 3 mol l^{-1} lithium acetate and 8 mmol l^{-1} KCl. The Corning 477317-filled barrel was used for monitoring the TMA^+ concentration and hence the cell volume. The reference barrel was used for recording E_m. The electrode barrels were connected to the inputs of an electrometer amplifier (FD223, WPI, Mauer, Germany) *via* chlorinated silver wires. The electrodes were calibrated before and after each experiment, and the two calibrations were applied to the recordings by linear interpolation. For cell volume measurements under voltage-clamp conditions, an additional single-barrelled electrode was inserted into the cell for current injection. The cells were loaded with TMA^+ by incubating the preparations in standard leech saline plus 5 mmol l^{-1} TMA^+ chloride for 5–10 min. TMA^+ is taken up by the cells at a rate of 0.3 mmol l^{-1} min^{-1}, and after removing TMA^+ from the bath solution the cells lose the substance at a rate that is 5–10 times smaller (Neumann et al., 2001).

Solutions

The standard leech saline (SLS) had the following composition (in mmol l^{-1}): 85 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes. The pH was adjusted to 7.40 with 1 mol l^{-1} NaOH, which increased the Na^+ concentration by 4 mmol l^{-1}. The osmolality of the SLS was 190 mosmol kg^{-1} H_2O (Osmomat 030, Gonotec, Berlin, Germany).

The composition of the anisotonic solutions corresponded to that of the SLS, except that the amount of NaCl was either reduced or increased to obtain relative osmolalities between ~20% (–81 mmol l^{-1} NaCl) and ~270% (+170 mmol l^{-1} NaCl), as referred to the SLS osmolality. In some experiments, we added sorbitol or glucose to the SLS to increase the osmolality by up to ~370% (+500 mmol l^{-1} sorbitol or glucose). In isotonic solutions with reduced Na^+ and/or Cl^- concentrations, Na^+ was replaced by $NMDG^+$ (*N*-methyl-D-glucammonium) and Cl^- by gluconate. In isotonic solutions with reduced ionic strength 40 mmol l^{-1} NaCl was replaced by 80 mmol l^{-1} sucrose.

DIDS (disodium 4,4′-diisothiocyanatostilbene-2,2′-disulphonate) was dissolved in 70% ethanol and added to the solutions at a final concentration of 0.5 mmol l^{-1} shortly before use. Colchicine (25 μmol l^{-1}), cytochalasine B and D (0.5 mmol l^{-1}), paclitaxel (30 nmol l^{-1}) and vinblastine (200 μmol l^{-1}) were added in the same way, but the cells were pre-incubated in the respective solutions for 1 h before the experiments began. NPPB [5-nitro-2-(3-phenylpropylamino) benzoic acid] was dissolved in DMSO and added to the solutions at a final concentration of 50 μmol l^{-1}. Propidium iodide (500 nmol l^{-1}) was dissolved in physiological solution. Cytochalasine D was purchased from Fluka (Buchs, Switzerland) and propidium iodide from Molecular Probes; all other substances were from Sigma.

Data analysis

The effect on E_m after changing the extracellular osmolality was quantified by subtracting the E_m value measured under anisotonic conditions from that measured before in SLS. Similarly, the effect on the membrane current was quantified by subtracting the current recorded in SLS at a given E_h from the corresponding current recorded under anisotonic conditions. The effect of the extracellular osmolality on R_{in} was quantified by normalizing the R_{in} value measured under anisotonic conditions to that measured before in SLS. The significance of the effects was tested using Student's paired two-tailed *t*-test. Differences were considered significant when $P<0.05$ (*) and highly significant when $P<0.01$ (**). Data are presented as means ± s.d.

RESULTS

Effect of anisotonic conditions on cell volume and the intracellular concentrations of Ca^{2+}, Na^+ and H^+

To investigate whether leech Retzius neurons possess volume-regulating mechanisms, we exposed the cells to anisotonic conditions. The shape of the cell bodies of the Retzius neurons was monitored with a confocal laser-scanning microscope (Fig. 1). Under control conditions, i.e. upon superfusion with isotonic SLS, the cell bodies were almost ball shaped. Under hypertonic conditions (+85 mmol l^{-1} NaCl), the cell volume decreased and the cells showed membrane invaginations. Under hypotonic conditions (–59 mmol l^{-1} NaCl), the cell volume increased and membrane evaginations or 'blebs' were observed. Cell swelling and shrinkage were reversible within 5 min. To exclude volume effects due to necrosis or apoptosis we applied propidium iodide, which would mark the nucleus after cell death (Haugland, 2002). However, such a staining was never observed.

The changes in cell volume upon varying the extracellular osmolality, as measured by using Fura-2 as a volume marker, are shown in Fig. 2. Cell shrinkage was sometimes followed by RVI (Fig. 2A): in +85 mmol l^{-1} NaCl, 24 out of a total of 84 cells showed RVI, during which the cell volume partly recovered from a minimum of 77±7% of the control value in SLS to 82±6%. In contrast, RVD after cell swelling was never observed (Fig. 2C, $N=75$, –40 to –81 mmol l^{-1} NaCl).

The volume changes of the Retzius neurons were smaller than predicted for an ideal osmometer, i.e. in the case of cell shrinkage, the measured volume was larger than expected, and in the case of cell swelling it was smaller (Fig. 2B,D). The volume of an ideal osmometer is inversely proportional to the osmolality of the

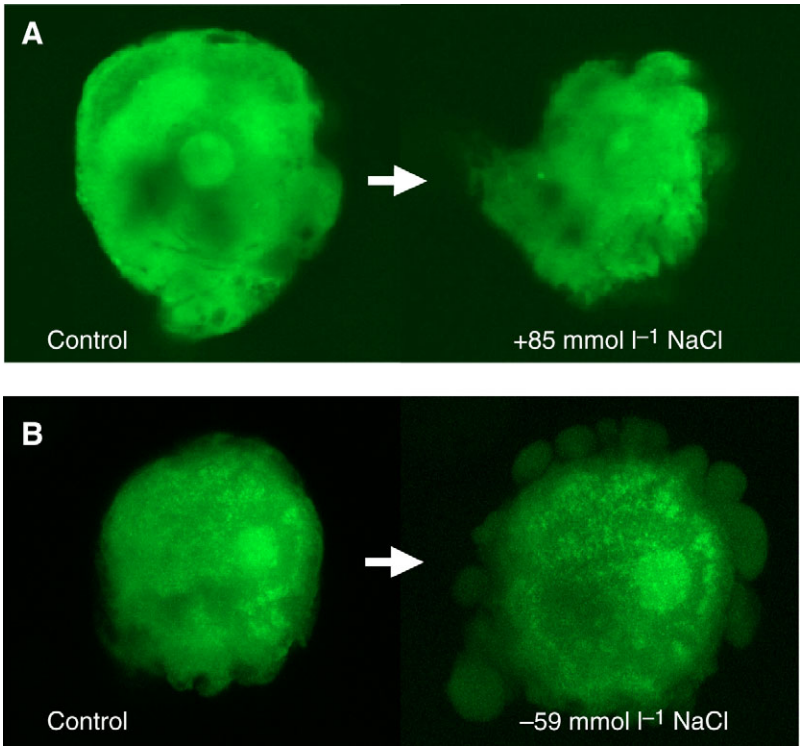

Fig. 1. Confocal images of leech Retzius neurons under isotonic and anisotonic conditions. Retzius neurons were loaded iontophoretically with the fluorescent dye Oregon Green® 488 BAPTA-1. Under isotonic conditions (Control), the cell bodies of the neurons were ball shaped. Under hypertonic conditions (+85 mmol l^{-1} NaCl, A), the neurons shrank and showed membrane invaginations. Under hypotonic conditions (–59 mmol l^{-1} NaCl, B), the neurons swelled and showed evaginations ('blebs'). Images were taken after 5 min under anisotonic conditions.

surrounding medium, and if Retzius neurons behaved in this way, one would expect $V_{exp}/V_0=\pi_0/\pi_{exp}$, where V_0 and V_{exp} are the cell volume at osmolality π_0 and π_{exp}, respectively, and the index '0' indicates isotonic conditions and 'exp' the experimentally changed, anisotonic conditions. Thus, for an ideal osmometer the plot of V_{exp}/V_0 against π_0/π_{exp} would deliver a straight line with a slope of 1 (Fig. 2B,D, broken line).

Monitoring cell volume changes with the fluorescent dyes Fura-2, BCECF and SBFI gave very similar results (not shown). Furthermore, the measured volume changes were similar to those

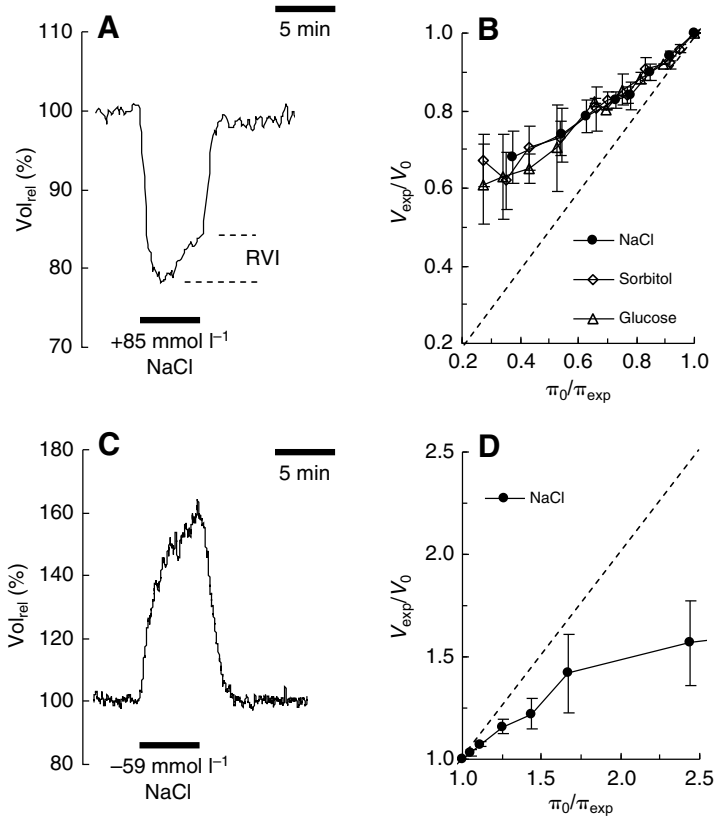

Fig. 2. Volume changes of Retzius neurons under anisotonic conditions. Volume changes were measured *via* the fluorescence intensity of Fura-2 excited at its isosbestic wavelength (see text for details). (A) Hypertonic solution (+85 mmol l^{-1} NaCl, ~0.5 π_0/π_{exp}) induced cell shrinkage. In 24 out of a total of 84 cells regulatory volume increase (RVI) was observed, during which the cell volume partly recovered from a minimum of 77±7% of the control value in standard leech saline (SLS) to 82±6%. (B) Raising the extracellular osmolality caused decreases in the cell volume that were significantly smaller than expected for an ideal osmometer (broken line). Note that the cell shrinkage was not dependent on whether the extracellular osmolality was increased by adding NaCl, which also raises the osmotic strength of the extracellular solution, or by adding carbohydrates such as sorbitol or glucose, which leaves the ionic strength constant. (C) Hypotonic solution (–59 mmol l^{-1} NaCl, ~2.4 π_0/π_{exp}) induced cell swelling. A regulatory volume decrease (RVD) was never observed (N=75, –40 to –81 mmol l^{-1} NaCl). (D) Reducing the extracellular osmolality caused cell volume increases that were significantly smaller than expected for an ideal osmometer (broken line). Vol_{rel}, relative cell volume. V_0 and V_{exp}, cell volume at osmolality π_0 and π_{exp}, with index '0' indicating isotonic conditions (SLS) and index 'exp' indicating experimentally changed, anisotonic conditions. The volume of an ideal osmometer is inversely proportional to the osmolality of the surrounding medium. This relationship is indicated by the broken line in B and D. Data in B and D are means ± s.d. of N=2–84 experiments.

Table 1. Effect of changing osmolality on intracellular pH and Ca^{2+} and Na^+ levels

	$[Ca^{2+}]_i$ (nmol l^{-1})	pH_i	$[Na^+]_i$ (mmol l^{-1})
SLS	77±9 (207)	7.3±0.2 (49)	7±3 (54)
+85 mmol l^{-1} NaCl	3±8 (53)	–0.08±0.11 (25)	5±3 (14)
–59 mmol l^{-1} NaCl	14±18 (58)	–0.02±0.09 (6)	–1±1 (13)

$[Ca^{2+}]_i$, pH_i and $[Na^+]_i$ under isotonic conditions (standard leech saline, SLS) and the change in these after increasing or reducing the extracellular osmolality (+85 mmol l^{-1} NaCl, –59 mmol l^{-1} NaCl). Data are means ± s.d.; number of experiments is given in parentheses.

recorded with ion-sensitive microelectrodes by using TMA^+ as a volume marker (Dierkes et al., 2002) (see Fig. 5). Intracellular Ca^{2+} concentration, pH and Na^+ concentration, measured in parallel with cell volume, remained largely unchanged (Table 1).

Effect of changing the extracellular NaCl concentration on E_m and R_{in}

Since volume changes under anisotonic conditions remained below those expected for an ideal osmometer, a transport of ions across the cell membrane during the swelling or shrinkage process is likely to have occurred. If this were indeed the case, the cell volume changes would depend on the membrane potential (E_m) or, *vice versa*, volume changes should cause changes in E_m. Furthermore, if ion channels were activated to mediate the ion transport, a change in the cell's input resistance (R_{in}) should be detected. To test this, we measured the effect of changing the extracellular osmolality on E_m and R_{in}. Upon superfusion of the preparations with SLS, the resting values of these two parameters were: E_m=–50.0±7.2 mV, R_{in}=8.3±1.7 MΩ (N=235 each). As shown in Fig. 3, a decrease in the extracellular osmolality, by reducing the NaCl concentration of the bath solution (–40 mmol l^{-1} NaCl), induced a membrane hyperpolarization (–5.1±5.3 mV, N=56) that was paralleled by an attenuation of action potential activity and by a decrease in R_{in} (–24±14%, see Fig. 4B). After returning to SLS, the cells transiently depolarized, which was accompanied by an enhanced action potential generation and a further R_{in} decrease, but subsequently all parameters recovered within 10 min. In contrast, an increase in the extracellular osmolality (+40 mmol l^{-1} NaCl) induced a membrane depolarization and a temporarily enhanced action potential activity. Initially, R_{in} decreased by ~20%, but then it recovered, and after a few minutes it was slightly larger than before the osmolality change (see Fig. 4B).

The changes in E_m and R_{in} induced by varying the extracellular osmolality between 42 and 190% of that of the SLS are summarized in Fig. 4. The effects were well reversible within 10 min, except for very low osmolalities of 50% or less, at which recovery was slow and mostly incomplete. The data show that the membrane depolarization under hypertonic conditions increased continuously with the extracellular osmolality, suggesting that it was predominantly mediated by the increase of the electrochemical Na^+ gradient (Fig. 4A). The hyperpolarization under hypotonic conditions was maximal at a moderately decreased osmolality and became smaller when the osmolality was further reduced, which indicates that it was not exclusively due to the reduction of the Na^+ gradient. The persistent R_{in} increase under hypertonic conditions was small (~10%), while the R_{in} decrease under hypotonic conditions was considerably more pronounced, particularly at osmolalities lower than ~50% (Fig. 4B). The changes in R_{in} suggest that, in leech Retzius neurons, shrinkage-activated ion channels are virtually absent, while swelling-activated ion channels may well exist. The following experiments were performed in order to characterize these channels in more detail.

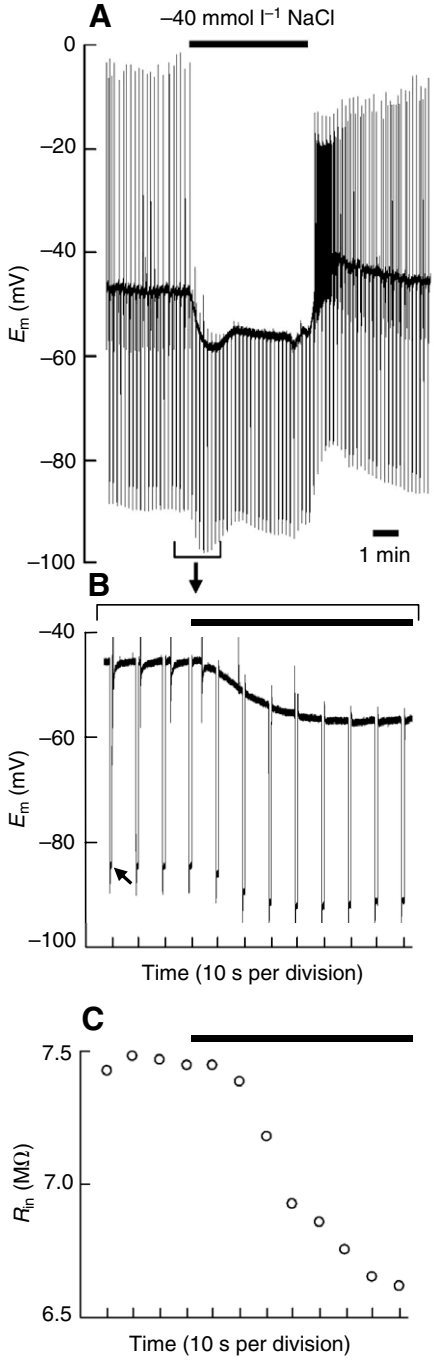

Fig. 3. Effect of reducing the extracellular NaCl concentration on E_m, R_{in} and the generation of action potentials. (A) Continuous recording showing the effect of reducing the extracellular NaCl concentration by 40 mmol l^{-1} for 5 min. Upward deflections are spontaneous action potentials, downward deflections were caused by the injection of negative current (–5 nA, duration 1 s, pulse interval 10 s). (B) Segment of the trace in A on an expanded time scale, shortly before and after the reduction of the extracellular NaCl concentration, as indicated. Action potentials truncated due to high gain. Note the partial recovery of the current-induced hyperpolarization ('depolarizing sag', arrow), which is due to the activation of hyperpolarization-activated (I_h) channels. (C) R_{in} calculated from the current-induced hyperpolarization shown in B.

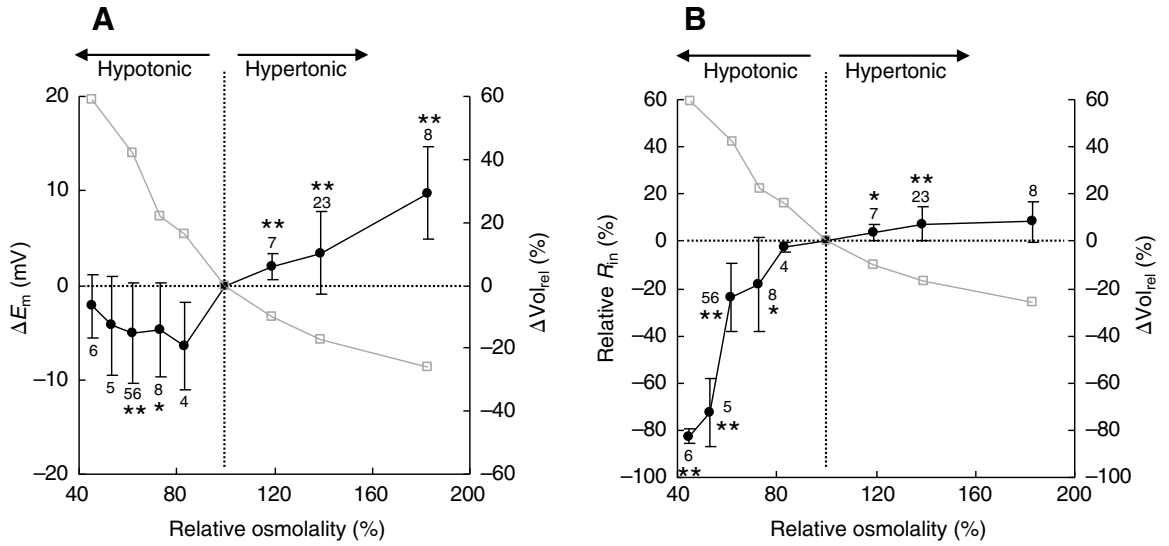

Fig. 4. Effect of the extracellular osmolality on E_m and R_{in}. The extracellular osmolality was varied by increasing or decreasing the NaCl concentration of the bath solution, and the changes in E_m (A) and R_{in} (B) were determined 5 min later. For comparison, volume changes (ΔVol_{rel}) as a percentage of control values are shown (grey squares, right-hand axes, mean values of N=2–84 experiments, see Fig. 2B,D). Data are means ± s.d.; number of experiments is given next to each data point. Asterisks indicate significant differences from the data in SLS (*P<0.05, **P<0.01). Osmolalities of the anisotonic solutions were normalized to SLS (Relative osmolality, see Fig. 2).

The R_{in} decrease in hypotonic solution is induced by cell swelling

Changes in E_m or in the electrochemical gradients for Na^+ or Cl^-, or the general reduction of the extracellular ionic strength, might contribute to the R_{in} decrease upon a reduction in the extracellular NaCl concentration. However, after replacing 40 mmol l^{-1} NaCl with 80 mmol l^{-1} sucrose, the cells hyperpolarized similarly compared with when in –40 mmol l^{-1} NaCl solution (–4.6±3.0 mV, N=9), but R_{in} remained unchanged (+0.5±5.9%). R_{in} also remained unchanged when 40 mmol l^{-1} Na^+ was replaced by $NMDG^+$ (N=9), 40 mmol l^{-1} Cl^- was replaced by gluconate (N=7) or 40 mmol l^{-1} NaCl was replaced by $NMDG^+$ and gluconate (N=9). Thus, the isotonic reduction of the extracellular concentrations of Na^+ and Cl^- had no effect on R_{in}, which demonstrates that the R_{in} decrease upon

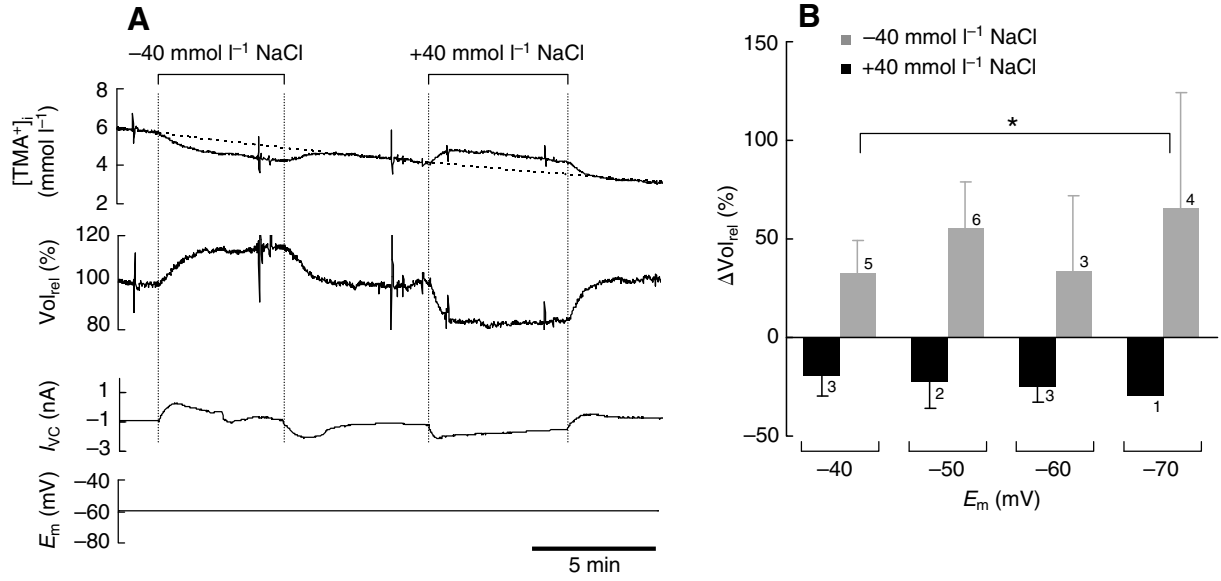

Fig. 5. Effect of the membrane potential on cell volume changes. (A) Cell volume was measured using ion-sensitive microelectrodes filled with Corning 477317, which monitored the concentration of the volume marker TMA^+. In parallel, E_m was clamped to a fixed holding potential (here –60 mV) and the clamp current (I_{VC}) was recorded. Hypotonic conditions (–40 mmol l^{-1} NaCl) caused a reversible swelling and a transient outward current, while hypertonic conditions (+40 mmol l^{-1} NaCl) caused a reversible shrinkage and a transient inward current. (B) Cell volume changes (ΔVol_{rel}) recorded under anisotonic conditions at E_m=–40 to –70 mV. Cell shrinkage did not change significantly with the holding potential, while cell swelling was almost twice as large at –70 mV as at –40 mV (P<0.05). Data are means ± s.d.; number of experiments is given beside the data bars (bar at +40 mmol l^{-1} NaCl, –70 mV results from a single experiment).

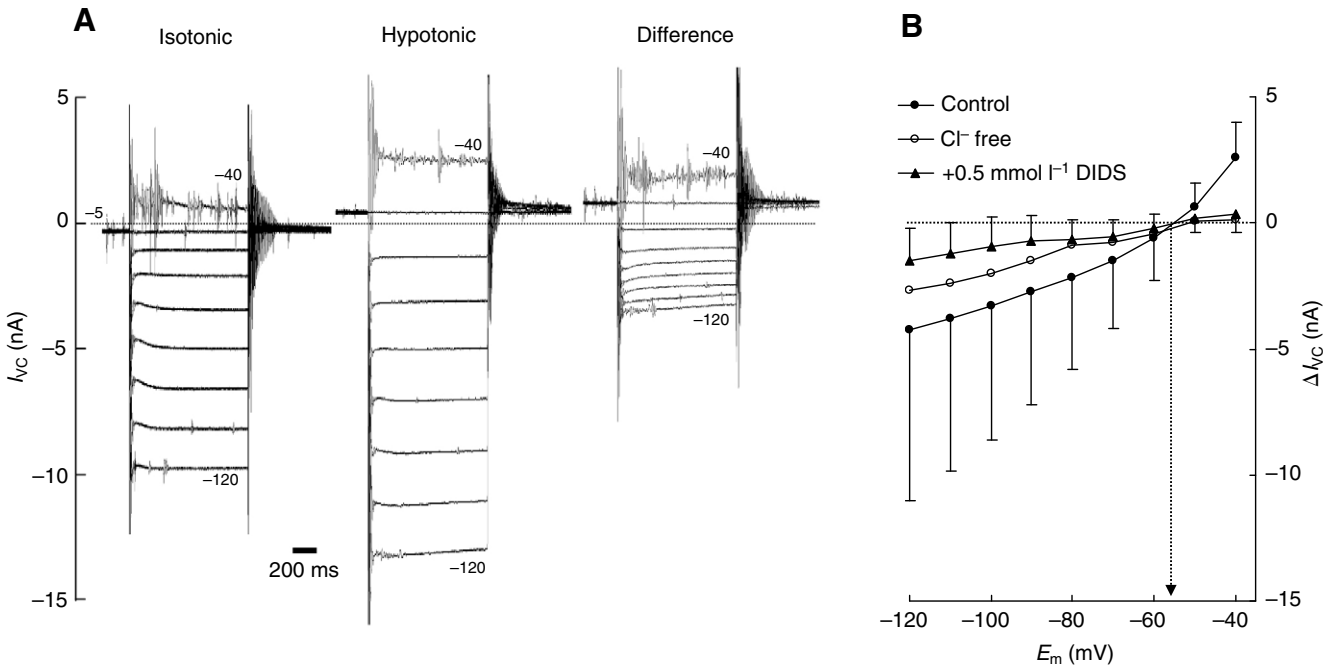

Fig. 6. Membrane currents under isotonic and hypotonic conditions. (A) Superimposed membrane currents upon shifting E_m for 1 s from –50 mV to values ranging from –120 to –40 mV (10 mV increments, 5 s intervals) under isotonic conditions (SLS) and after 5 min in hypotonic solution (–40 mmol l^{-1} NaCl). Subtraction of the current needed under isotonic conditions from the respective current under hypotonic conditions isolated the swelling-activated membrane current ('Difference'). The dependence of this current on E_m is shown in B. The traces are means of 34 experiments. Before averaging, the single traces were filtered through an 8-pole Bessel filter (100 Hz). The performance of the voltage-clamp protocol took ~1 min; in the meantime, the recording system was in the current-clamp mode. (B) Voltage dependence of the averaged swelling-activated membrane current under control conditions (see A), in Cl^-–free solution and in the presence of 0.5 mmol l^{-1} DIDS. Data are means ± s.d. of N=47 (control, included are 13 experiments with a clamp duration of only 0.5 s), N=7 (Cl^- free) and N=11 experiments (DIDS). Arrowhead indicates the reversal potential of the swelling-activated current under control conditions.

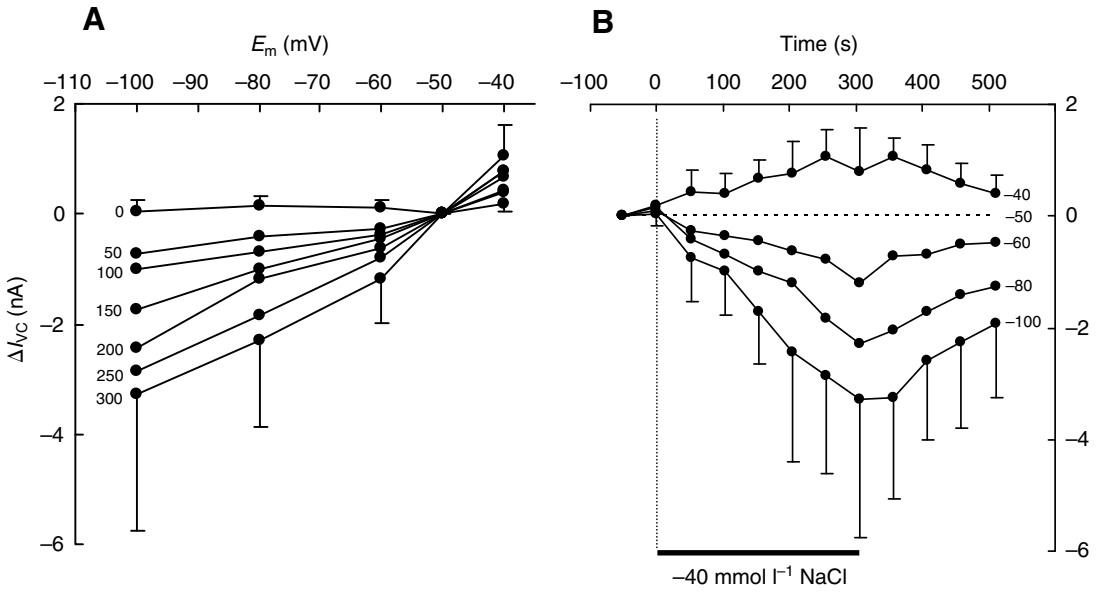

Fig. 7. Time course of activation of the swelling-induced membrane current. (A) Dependence of the swelling-activated membrane current on E_m at different times after reducing the extracellular osmolality (–40 mmol l^{-1} NaCl). Retzius neurons were clamped from a holding potential of –50 mV to –100, –80, –60 or –40 mV for 1 s with an interstimulus interval of 2 s (see Fig. 6A). This protocol was applied in SLS as well as 50, 100... 300 s after changing the extracellular osmolality to obtain the presented I–V relationships. Data are means ± s.d. of N=5 experiments. (B) Time course of the activation and deactivation of the swelling-induced membrane current, as derived from the data shown in A, as well as from current recordings after return to SLS. Activation and deactivation of the swelling-induced current occurred with a similar time course to the changes in cell volume (compare Fig. 2C, Fig. 5A, Fig. 10A,B).

reducing extracellular NaCl without osmotic compensation was predominantly due to cell swelling.

Volume changes under voltage-clamp conditions

In order to determine the role of E_m in the volume changes under anisotonic conditions, we combined cell volume measurements with ion-sensitive microelectrodes (using TMA^+ as a volume marker) with the voltage-clamp technique (Fig. 5). When E_m was held at its resting value (–50 mV), the detected volume changes were similar to those under unclamped conditions (see Dierkes et al., 2002). The volume changes at different holding potentials are summarized in Fig. 5B. Under hypertonic conditions, no voltage dependence was observed. Under hypotonic conditions, however, when clamping to –70 mV, the cell swelling was markedly increased, and it was reduced when clamping to –40 mV. Possibly, at –70 mV, the efflux of K^+ and/or Cl^- is prevented, since this holding potential is close to the K^+ equilibrium potential in these cells (E_K, approximately –75 mV) and more negative than the Cl^- equilibrium potential (E_{Cl}, approximately –60 mV) (Dierkes et al., 2002; Klees, 2005; Munsch et al., 1995; Munsch and Schlue, 1993). *Vice versa*, at –40 mV, the efflux of K^+ and Cl^- might be enhanced.

Swelling-activated membrane current

The opening of swelling-activated ion channels should be accompanied by changes in the membrane current. Indeed, after 5 min in hypotonic solution, the currents needed to clamp E_m to values between –120 and –40 mV were increased over the entire voltage range (Fig. 6A, left and middle traces). After returning to SLS, the membrane currents reached their initial values within 10 min. The swelling-activated current was isolated by subtracting the current needed in SLS to clamp E_m to a given value from the corresponding current necessary under hypotonic conditions (Fig. 6A, right traces). The dependence of the swelling-activated current on E_m (*I–V* relationship) showed slight outward rectification and the reversal potential of the current was –57±12 mV (N=47, Fig. 6B), which is close to E_{Cl} under control conditions. We note that the reduction of the extracellular NaCl concentration leads to a shift of E_{Cl} to a more positive value (+14 mV), but this effect would be largely counteracted by the subsequent decrease in the cytosolic Cl^- concentration due to cell swelling and Cl^- efflux. After a volume increase of ~57% (Dierkes et al., 2002) (see Fig. 5B), the cytosolic Cl^- concentration was calculated as ~5 mmol l^{-1}, which results in an E_{Cl} of approximately –60 mV, similar to E_{Cl} under control conditions.

Further evidence for the view that the additional membrane current in hypotonic solution is due to cell swelling was obtained by comparing the kinetics of current activation and cell swelling. The *I–V* relationship of the swelling-activated current at several time points after changing to hypotonic bathing solution is plotted in Fig. 7A. The swelling-activated current gradually increased during a 5 min exposure to hypotonic solution, did not reach a plateau within this time, but immediately began to decline after isotonic conditions were restored (Fig. 7B). This time course is similar to that of the cell volume changes (Fig. 2C, Fig. 5A, Fig. 10A,B).

Effect of cell swelling in Cl^-–free solution

The close correspondence between reversal potential and E_{Cl} suggests that the swelling-activated membrane current in leech Retzius neurons is predominantly mediated by Cl^- channels. To find further evidence for such swelling-activated Cl^- channels we investigated the effect of reducing the extracellular osmolality on R_{in} and on the membrane current in the absence of extracellular Cl^-. Under these conditions, Retzius neurons lose their cytosolic Cl^- within 5 min, probably due to a tonic Cl^- conductance (Beck et al., 2001; Klees, 2005; Munsch et al., 1995; Munsch and Schlue, 1993), so that an activation of Cl^- channels should have no effect on R_{in} or on the membrane current. The complete exchange of extracellular Cl^- for gluconate evoked a persistent membrane depolarization by 5–10 mV, paralleled by an enhanced generation of action potentials and, after some time, transient stronger depolarizations that were superimposed by bursts of action potentials (Beck et al., 2001; Munsch et al., 1995). Shortly after Cl^- removal, R_{in} decreased by ~30%, possibly due to the activation of voltage-dependent ion channels, but subsequently R_{in} recovered, and after a few minutes it was ~10% larger than in SLS, which may reflect the extinction of the tonic Cl^- conductance. Now, reducing the extracellular osmolality had virtually no effect on E_m and caused only a small reduction in R_{in} (see Fig. 8). The swelling-activated membrane current was also reduced in Cl^--free solution, whereby the reduction of the inwardly directed current was relatively small, while the outward current was virtually abolished (Fig. 6B).

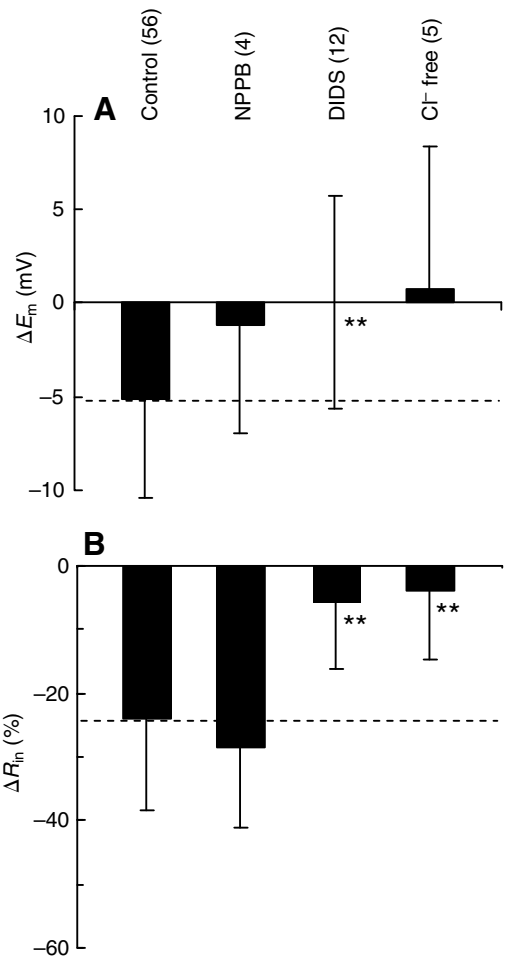

Fig. 8. Effect on E_m and R_{in} of reducing the extracellular osmolality in the presence of Cl^- channel blockers and in Cl^--free solution. The preparations were exposed to the Cl^- channel blocker DIDS (0.5 mmol l^{-1}) or NPPB (50 μmol l^{-1}), or to isotonic Cl^--free solution (Cl^- replaced by gluconate), and after 5 min E_m and R_{in} were measured. Subsequent reduction of the extracellular osmolality by omitting 40 mmol l^{-1} NaCl or sodium gluconate from the bath solution caused changes in E_m (A) and R_{in} (B) that were determined after 5 min. Significant differences from the control data (taken from Fig. 4, broken line) are indicated by asterisks (**P<0.01).

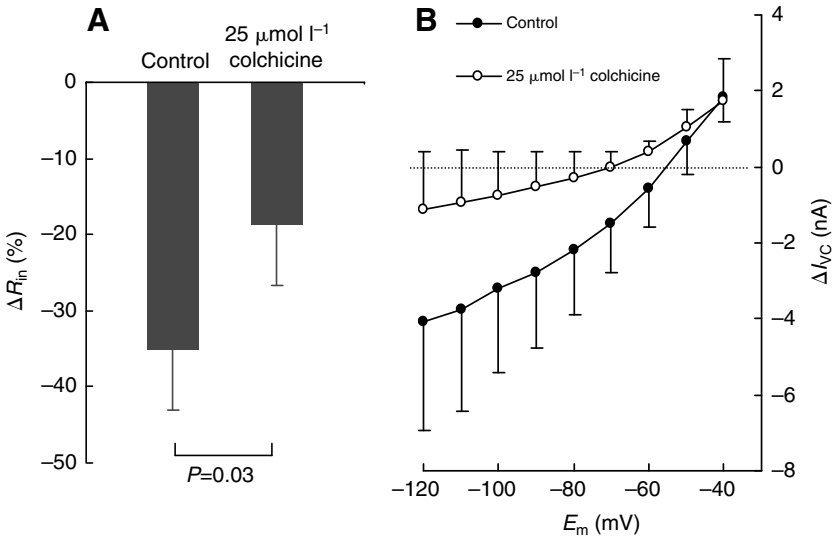

Fig. 9. Effect of colchicine on the swelling-induced changes in R_{in} and membrane current. The swelling-induced changes in R_{in} (A) and membrane current (B) were measured 5 min after reducing the extracellular osmolality (–40 mmol l^{-1} NaCl), either under control conditions or after incubation of the preparations in SLS plus 25 μmol l^{-1} colchicine for 1 h. After colchicine application the swelling-induced changes in R_{in} and membrane current were reduced. Data are means ± s.d. of N=5 experiments.

Effect of Cl^- channel blockers

In Cl^--containing solution, R_{in} and membrane current should be unchanged by reducing the extracellular osmolality if the Cl^- channels are blocked. The Cl^- channel blocker DIDS (0.5 mmol l^{-1}) induced a persistent, albeit very slight membrane depolarization, which was accompanied by an enhanced action potential activity (not shown). Furthermore, DIDS induced a persistent increase in R_{in} of ~20%, which may again reflect the extinction of the tonic Cl^- conductance. That the R_{in} increase was larger than in Cl^--free solution might be attributed to voltage-dependent ion channels activated by the larger membrane depolarization after Cl^- omission. In the presence of DIDS the reduction of the extracellular osmolality had no effect on E_m and diminished R_{in} only slightly (see Fig. 8). Furthermore, the swelling-activated membrane current was strongly reduced and, again, as in Cl^--free solution, the outward current was more affected than the inward current (Fig. 6B). In contrast to DIDS, the Cl^- channel blocker NPPB (50 μmol l^{-1}) had no effect on the R_{in} decrease upon reduction of the extracellular osmolality, while the membrane hyperpolarization was reduced (Fig. 8). The effect of NPPB on the membrane current was not investigated.

In the presence of Cl^- channel blockers, an increase in cell swelling might be expected, but neither DIDS (0.5 mmol l^{-1}) nor NPPB (200 μmol l^{-1}) had an effect (not shown). The effect of Cl^--free conditions on hypotonic cell swelling was not investigated, but the removal of Cl^- by itself did not affect the cell volume (Dierkes et al., 2006), and a swelling induced by inhibiting the Na^+–K^+ pump with ouabain, by activating ionotropic glutamate receptors with kainate, or by high extracellular K^+ (30 mmol l^{-1}) was unchanged in Cl^--free solution (Dierkes et al., 2006; Trosiner, 2003).

Taken together, the results show that Cl^- removal and Cl^- channel blockade by DIDS substantially attenuate the decrease in R_{in} and the increase in membrane current in response to reducing the extracellular osmolality. This supports the view that the swelling of leech Retzius neurons leads to the activation of Cl^- channels.

Cytoskeleton

In many preparations the cytoskeleton is involved in the control of swelling-activated ion channels, often *via* actin filaments (Fürst et al., 2002). In some cases, however, especially in leech mechanosensitive ion channels, the control seems to be exerted primarily by the microtubule system (Menconi et al., 2001). After exposure to the tubulin polymerization inhibitor colchicine, the effect of lowering the extracellular osmolality on R_{in} and on the membrane current was significantly reduced (Fig. 9). The reversal potential of the residual swelling-activated current was shifted by approximately –15 mV (Fig. 9B). This may be explained by an increased drop in the cytosolic Cl^- concentration which, in turn, may be due to the increased cell swelling (see Fig. 10A). The observations suggest that the swelling-activated Cl^- channels of leech Retzius neurons require an intact microtubule system in order to become appreciably activated.

After incubation with colchicine or vinblastine, which also inhibits tubulin polymerization, the cell swelling in hypotonic solution was approximately twice as large as under control conditions, while cell shrinkage was much less affected (Fig. 10A,B). The effects of both substances were completely abolished in the presence of the tubulin polymerization enhancer paclitaxel. In contrast, incubation with the actin polymerization inhibitors cytochalasin B or D had virtually no effect on the osmotically induced volume changes (Fig. 10C). These findings suggest the involvement of the microtubule system, but not of the actin system, in limiting cell swelling. Simultaneously, the cytosolic Ca^{2+} concentration was measured and found to be largely unchanged.

DISCUSSION

Most animal cells cannot sustain osmotic gradients across their cell membrane and, therefore, changes in the extracellular osmolality lead to changes in cell volume, which may affect various cellular functions (for reviews, see Lang et al., 1998; Pasantes-Morales et al., 2006). In general, cell volume changes are counteracted by specific cellular mechanisms, which cause RVD after cell swelling or RVI after cell shrinkage. Leech Retzius neurons only occasionally showed RVI and never RVD (Fig. 2). A similar behaviour has been shown previously for hippocampal neurons, which exhibited RVD only occasionally in slice preparations (Kreisman and Olson, 2003), and never in cultured neurons (Aitken et al., 1998). However, the volume changes of Retzius neurons upon changing the extracellular osmolality were distinctly smaller than expected for an ideal osmometer (Fig. 2B,D), suggesting that volume change and

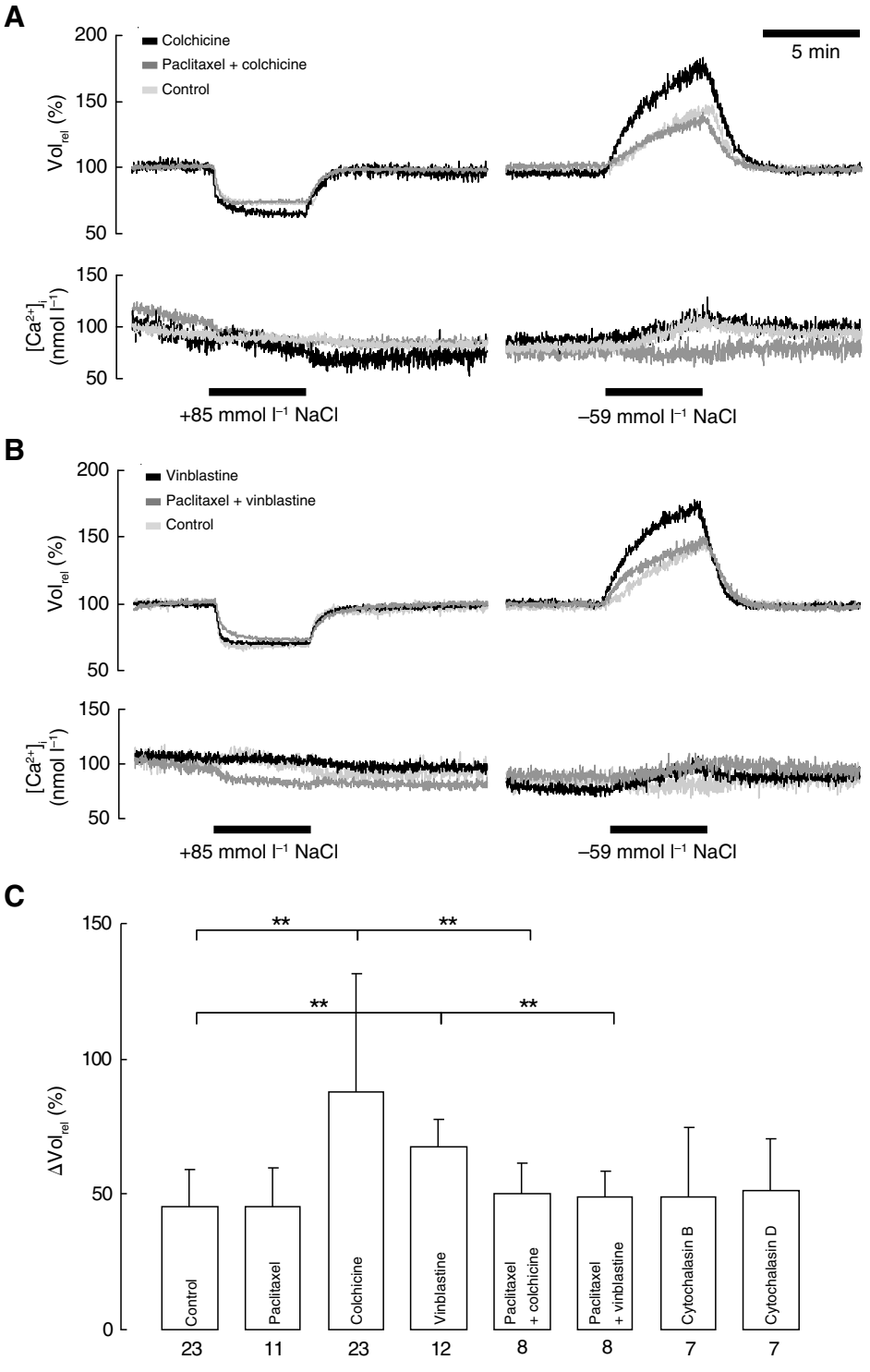

Fig. 10. Role of the cytoskeleton in cell volume changes. Using Fura-2 as a volume marker, cell volume changes were monitored in the presence of substances that affect the assembly of microtubules or actin filaments. (A,B) The tubulin polymerization inhibitors colchicine (25 μmol l^{-1}) and vinblastine (200 μmol l^{-1}) had little effect on the cell shrinkage under hypertonic conditions (+85 mmol l^{-1} NaCl) but caused an increased swelling under hypotonic conditions (–59 mmol l^{-1} NaCl; 5 min each). The effects were abolished in the presence of the tubulin polymerization enhancer paclitaxel (30 nmol l^{-1}). The simultaneously measured cytosolic Ca^{2+} concentration was hardly affected by the various drugs and/or changes in the extracellular osmolality. (C) Statistical analysis (means ± s.d., number of experiments below data bars) of experiments under hypotonic conditions with colchicine, vinblastine, paclitaxel, and the actin polymerization inhibitors cytochalasine B and D. Cell swelling increased when tubulin polymerization was inhibited (P<0.01) but not when actin polymerization was inhibited.

volume regulation occur more or less in parallel (Dierkes et al., 2002; Dierkes et al., 2003).

Several physiological and pathophysiological conditions may change the haemolymph osmolality of the medicinal leech. During ingestion, leeches take up 10–15 times their own body weight of blood (Nieczaj and Zerbst-Boroffka, 1993). This is accompanied by an elevation of the osmolality of the haemolymph, which directly affects the central nervous system located within the ventral blood sinus of the animal. The resulting cell shrinkage might, in part, be counteracted by the (occasionally) observed RVI (Fig. 2A). On the other hand, leeches completely change their behaviour after blood ingestion (Dickinson and Lent, 1984), and one may speculate whether the hypertonicity of the haemolymph contributes to this.

Cell swelling may occur under conditions that lead to a high extracellular K^+ concentration, which causes a membrane depolarization and hence favours Cl^- uptake. Increases in the extracellular K^+ concentration up to 10 mmol l^{-1} were observed after high neuronal activity in the leech and in other preparations (Baylor and Nicholls, 1969; Dietzel et al., 1989), up to

~50 mmol l^{-1} during hypoxia (Müller and Somjen, 2000), and up to ~80 mmol l^{-1} during spreading depression (Mazel et al., 2002). In Retzius neurons, an experimental increase of the extracellular K^+ concentration causes cell swelling by means of an electroneutral KCl uptake (Neumann et al., 2001; Trosiner, 2003). Under these conditions, the cytosolic Cl^- concentration increases and swelling-activated Cl^- channels would help to restore cell volume and cytosolic Cl^- concentration during the recovery process.

Swelling-activated Cl^- channels in leech Retzius neurons

The decrease in R_{in} upon reduction of the extracellular osmolality (Figs 3, 4 and 8) and the increase in the membrane current (Figs 6 and 7) both indicate that leech Retzius neurons activate ion channels in response to cell swelling. The reversal potential of the swelling-activated current was close to E_{Cl}, which suggests that the changes in R_{in} and membrane current were mediated by Cl^- channels. Furthermore, the swelling-activated membrane current showed slight outward rectification (Fig. 6), which is typical for swelling-activated Cl^- channels (Fürst et al., 2002). Finally, the swelling-induced decrease in R_{in} and the increase in membrane current were substantially attenuated by the removal of extracellular Cl^- or in the presence of the Cl^- channel blocker DIDS (Figs 6 and 8). Under both conditions, the changes in R_{in} and membrane current were not abolished, which in the case of DIDS might be explained by an incomplete blockade of the swelling-activated Cl^- channels. In the absence of extracellular Cl^-, however, the persistence of a Cl^- current is unlikely, because cytosolic Cl^- is rapidly washed out (Beck et al., 2001; Klees, 2005; Munsch et al., 1995; Munsch and Schlue, 1993). As Cl^- channels are permeable to many small anions (Fürst et al., 2002; Okada, 1997), the residual changes in R_{in} and membrane current may be explained by an augmented flux of anions that occur naturally in the cytosol, or of sulphate ions that are delivered by the recording electrodes, or of gluconate ions that were used to replace Cl^-.

The swelling-activated Cl^- channels of leech Retzius neurons are unusual in that they are not affected by NPPB (Fig. 8), which effectively blocks swelling-activated Cl^- channels in most other preparations (see Barrière et al., 2003; Fürst et al., 2002; Okada, 1997; Wehner et al., 2003). Cl^- channels that are insensitive to NPPB have been described previously; however, these channels of the ClC-3 or ClC-5 type are not activated by osmotic swelling (Li et al., 2000; Kong et al., 2006). NPPB-insensitive 'Cl^--dependent Cl^- channels', which increase their open probability after a drop in the extracellular Cl^- concentration, were found in leech peripheral neurons ('nephridial nerve cells') (Wenning et al., 2001). However, these channels were also insensitive to DIDS. Furthermore, in Retzius neurons, the isotonic reduction of the extracellular Cl^- concentration had no effect on R_{in}, which argues against the activation of a Cl^--dependent conductance. The swelling-activated Cl^- channels described here may be functionally similar to those found in other preparations, albeit they appear pharmacologically distinct and show a particular activation mechanism. Thus, further studies are needed to characterize these channels in more detail.

The swelling-activated Cl^- channels may significantly raise the Cl^- conductance of the cell membrane. Under isotonic conditions, the Cl^- conductance comprises ~20% of the total membrane conductance, as indicated by the DIDS-induced increase in R_{in} and by the rate of change in the cytosolic Cl^- concentration after changing the extracellular Cl^- concentration (Klees, 2005). According to the decrease in R_{in} (Figs 3 and 4), as well as the increase in membrane current (Fig. 6), reducing the extracellular NaCl concentration by 40 mmol l^{-1} causes an increase in the total membrane conductance by ~30%. Provided that this increase is exclusively due to the swelling-activated Cl^- channels, the Cl^- conductance of the cell membrane is increased by a factor of 2.5.

In various leech neurons, stretch-activated cation channels have been found (Calabrese et al., 1999; Menconi et al., 2001; Pellegrino et al., 1990), but it is unlikely that these channels contribute significantly to the changes in R_{in} and membrane current described here: the channels are permeable to K^+, Na^+ and Ca^{2+} (Calabrese et al., 1999) and, therefore, channel activation should induce a membrane depolarization as well as a membrane current with a reversal potential more positive than the resting E_m, which is incompatible with our experimental data (Figs 3, 4 and 6). Furthermore, channel activation should cause an influx of Na^+ and Ca^{2+}, but reducing the extracellular NaCl concentration by up to 59 mmol l^{-1} had virtually no effect on the cytosolic concentrations of Na^+ and Ca^{2+} (Table 1, Fig. 10A,B).

Physiological relevance

Upon omitting NaCl from the bathing solution the cells swelled less than expected for an ideal osmometer (Fig. 2D) (see Dierkes et al., 2002). This discrepancy could be due to the release of osmolytes from the cell, to which the swelling-activated Cl^- channels may contribute, provided that E_{Cl} becomes more positive than E_m. This will surely be the case immediately after reducing the extracellular NaCl concentration, because the cells hyperpolarize and E_{Cl} is shifted towards more positive values. In the course of time, however, E_{Cl} is shifted back to its original value, because the cytosolic Cl^- concentration drops, due to both cell swelling and Cl^- release, i.e. the electromotive force driving Cl^- efflux decreases. Yet, with increasing cell volume, the activity of the swelling-activated Cl^- channels will increase, which helps to maintain Cl^- release and thus limits the swelling of the cell. Nevertheless, the osmotic effect of Cl^- release is small, as even the efflux of all cytosolic Cl^- [~10 mmol l^{-1} at rest (Dierkes et al., 2002; Klees, 2005; Munsch et al., 1995; Munsch and Schlue, 1993)], together with K^+ as a counter ion, would reduce the cytosolic osmolality by only 10%. Therefore, it is unlikely that the physiological function of the swelling-activated Cl^- channels is to limit the cell swelling after a drop in the extracellular osmolality. Indeed, leech neurons do not encounter hypotonicity under physiological conditions. In its natural freshwater environment, the medicinal leech is protected by its cuticula and the secreted mucus, and the animal is able to tolerate permanent exposure to a hypotonic environment with the central nervous system being unaffected (Zerbst-Boroffka, 1973).

Instead, the swelling-activated Cl^- channels might well be involved in cell volume regulation and ion homeostasis under physiological conditions, particularly after periods of high neuronal activity. Neuronal activity is expected to induce an uptake of NaCl and hence to cause an increase in both cytosolic Cl^- concentration and cell volume (Dierkes et al., 2006; Neumann et al., 2001). The increase in cell volume may affect a variety of cellular functions, while the increase in the cytosolic Cl^- concentration will specifically attenuate or even inverse the effect of inhibitory synaptic input. In this situation, the swelling-activated Cl^- channels should facilitate the release of Cl^- from the cytosol, thereby accelerating the restoration of both cell volume and cytosolic Cl^- concentration.

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