J. exp. Biol. **163**, 169–186 (1992)
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THE IONIC BASIS OF THE HYPO-OSMOTIC DEPOLARIZATION IN NEURONS FROM THE OPISTHOBRANCH MOLLUSC *ELYSIA CHLOROTICA*

BY R. H. QUINN* AND S. K. PIERCE
Department of Zoology, University of Maryland, College Park, MD 20742, USA

Accepted 15 October 1991

Summary

The resting potential of identified cells (Parker cells) in the abdominal ganglion of *Elysia chlorotica* (Gould) depolarizes by about 30 mV in response to a 50 % reduction in osmolality and returns to the original potential in 20 min. Cell volume recovery requires approximately 2 h. Thus, recovery of the resting potential is not dependent on recovery of cell volume. The hypo-osmotic depolarization persists following inhibition of the electrogenic Na^+/K^+-ATPase with ouabain, and the levels of extracellular K^+ and Cl^- have little effect on the magnitude of the depolarization, while decreasing extracellular Na^+ concentration produces a depolarization of only 10 mV. This suggests that the hypo-osmotic depolarization in Parker cells results mostly from increased relative permeability to Na^+. Following transfer from 920 to 460 mosmol kg^{-1}, Na^+, Cl^- and proline betaine leave the cells while intracellular K^+ is conserved. Loss of intracellular Na^+ and conservation of intracellular K^+ are dependent on active transport by the Na^+/K^+-ATPase. Na^+ and proline betaine leave the cells with a time course that is much longer than that of the hypo-osmotic depolarization. Unlike the other solutes, most of the reduction in intracellular Cl^- concentration occurs coincidentally with the hypo-osmotic depolarization. However, unlike the hypo-osmotic depolarization, bulk loss of Cl^- does not require the reduction in osmolality, only the reduction in extracellular ion concentrations. There is no apparent relationship between membrane depolarization and the regulation of intracellular osmolytes in *Elysia* neurons following hypo-osmotic stress.

Introduction

In response to hypo-osmotic stress, membrane potential changes occur in some but not all cell types. For example, a hypo-osmotic stress produces a membrane depolarization (sometimes preceded by a brief hyperpolarization) in *Sabella penicillus* giant axon (Treherne and Pichon, 1978), *Mytilus edulis* cerebrovisceral connectives (Willmer, 1978*b*), *Mya arenaria* neurons (Beres and Pierce, 1981),

* Present address and address for correspondence: 360 Minor Hall, U.C. Berkeley, Berkeley, CA 94720, USA.

Key words: hypo-osmotic, membrane depolarization, neurons, *Elysia chlorotica*.

Limulus polyphemus cardiac ganglion follower cells (Prior and Pierce, 1981), rat brain astrocytes (Kimelberg and O'Connor, 1988), Madin–Darby canine kidney cells (Volkl *et al.* 1988), Ehrlich ascites tumor cells (Lambert *et al.* 1989) and opossum kidney (OK) cell line (Ubl *et al.* 1989). Following depolarization, the membrane potential repolarizes back to, or slightly below, the original resting potential (Prior and Pierce, 1981). In contrast, hypo-osmotic stress hyperpolarizes *Necturus maculosus* small intestine (Lau *et al.* 1984) and mouse hepatocytes (Howard and Wondergem, 1987). However, no membrane potential change occurs in *Callinectes sapidus* walking leg muscle (Lang and Gainer, 1969) or *Amphiuma means tridactylum* blood cells (Cala, 1980) in response to the stress.

The hypo-osmotically induced depolarization does not occur when ions alone are reduced in the absence of an osmotic change (Carlson *et al.* 1978; Prior and Pierce, 1981). Thus, the reduction in osmolality and not the reduction in extracellular ion concentrations causes the hypo-osmotic depolarization. The permeability changes that result in osmolyte loss and volume recovery following hypo-osmotic stress also result from the reduction in osmolality rather than in extracellular ion concentrations (e.g. human red cells, Poznansky and Soloman, 1972; bivalve ventricles, Pierce and Greenberg, 1973; Ehrlich ascites tumor cells, Hendil and Hoffman, 1974; flounder erythrocytes, Fugelli and Rohrs, 1980; sheep red cells, Dunham and Ellory 1981; flounder ventricles, Vislie, 1983). Thus, both the hypo-osmotic depolarization and the permeability changes that control osmolyte efflux and cell volume regulation result from the same stimulus.

However, a causal relationship between the membrane potential change and solute efflux during hypo-osmotic stress has only been established in three cell types. Hypo-osmotic stress activates a conductive efflux of Cl^- and K^+ in human lymphocytes (Grinstein *et al.* 1982), chinese hamster ovary cells (Sarkadi *et al.* 1984) and Ehrlich ascites tumor cells (Lambert *et al.* 1989). The hypo-osmotically activated Cl^- permeability is greater than that for K^+, resulting in membrane depolarization (human lymphocytes, Grinstein *et al.* 1982; chinese hamster ovary cells, Sarkadi *et al.* 1984). Similarly, the hypo-osmotic depolarization in *Mytilus edulis* connectives results from increased permeability to Cl^- (Willmer, 1978*b*); however, a relationship to solute loss was not established.

The purpose of this study was to determine whether the hypo-osmotic depolarization in neurons results from a volume-regulating efflux of ions. The ionic basis of the hypo-osmotic depolarization was examined to identify which ions are involved, the levels of intracellular osmolytes were measured to determine which ones are responsible for cell volume regulation, and the time courses of the depolarization and solute loss were compared to that of cell volume recovery to test for correlation between the events.

Ganglia from an opisthobranch mollusc, *Elysia chlorotica*, were chosen for this study for several reasons. *Elysia* is an extremely euryhaline osmoconformer; blood osmolarity parallels environmental osmolarity over a wide range (Pierce *et al.* 1983). Thus, the individual cells from any tissue in this animal must be extremely capable volume regulators. Also, the abdominal ganglion contains a pair of

identified somas, the Parker cells (Parker and Pierce, 1985), which are well suited for electrophysiological measurements. Since the cells are uniquely identifiable, the same cells could be utilized in every animal, thus reducing the variability that might be encountered by using random cells. The large size of these cells (approximately 100 μm in diameter) permits easy microelectrode penetration for intracellular recording, and impalement is usually not dislodged by cell swelling following a change in osmolality from 920 to 460 mosmol kg^{-1}. Parker cells are very tolerant of hypo-osmotic stress and maintain excitability even after abrupt dilution from 100 % to 25 % sea water. Also, the large size of these cells allows for optical measurement of cell volume changes. Like most cells following hypo-osmotic stress, Parker cells swell and then partially recover their initial cell volume. Finally, like other neurons, Parker cells transiently depolarize in response to hypo-osmotic stress.

Materials and methods

Maintenance of animals

Elysia chlorotica were collected from a salt marsh near Menemsha on Martha's Vineyard Island, MA. The animals were maintained in Instant Ocean sea water at 920 mosmol kg^{-1} and 10°C on a 15 h dark, 9 h light cycle. Animals were allowed to come to room temperature just prior to an experiment, and all experiments were run at room temperature, 21.6±1.5°C (mean±standard deviation).

Solutions

Artificial seawater (ASW) solutions with differing ionic contents were mixed from stock solutions of individual, reagent grade salts using formulae adapted from Costa and Pierce (1983) buffered to pH 7.8 (Table 1). Concentrations of individual ionic species in the ASW were manipulated by substituting equimolar amounts of one salt for another as indicated. Quinidine and ouabain were obtained from Sigma.

Membrane potential measurements

Circumesophageal nerve rings were dissected from animals that had been acclimated to 920 mosmol kg^{-1} for at least 5 days. The ring of ganglia was transferred to a bathing chamber containing 920 mosmol kg^{-1} ASW and secured to the Sylgard bottom of the chamber by covering it with a nylon mesh held by glass pins. The chamber allowed for rapid superfusion of the tissue. During ion substitutions, the tissues were exposed to the ion-substituted ASW at 920 mosmol kg^{-1} for 5 min before perfusion with ion-substituted ASW at 460 mosmol kg^{-1}.

Membrane potentials were recorded intracellularly from one of an identified pair of pigmented somas, Parker cells, on the abdominal ganglion (Parker and Pierce, 1985) by impalement with 3 mol l^{-1} KCl-filled glass micropipettes. Electrical connection to the KCl-filled pipettes was made by way of a Ag–AgCl pellet

Table 1. *Ionic content (mmol l^{-1}) of artificial sea water formulae at 920 mosmol kg^{-1}*

Formula name	Na^+	K^+	Cl^-	Ca^{2+}	Mg^{2+}	SO_4^{2-}	HCO_3^-	$Tris^+$	Arg^+	MS^-	Mops
Normal ASW	426	8.9	496	9.9	47	25	2.3				5
Na^+-free		8.9	423	9.9	47	25		489			
Ca^{2+}-free*	441	8.9	492		47	25	2.3				5
Na^+:Ca^{2+}-free*		8.9	439		47	25		503			
Na^+-free (Arg^+)		8.9	498	9.9	47	25		~5	426		
Na^+-free:low-Cl^- (sucrose)		8.9	106	9.9	47	25		49			
Na^+-free:low-Cl^- (TrisMS)		8.9	106	9.9	47	25		422		331	
Na^+:Ca^{2+}-free:low-Cl^- (sucrose)*		8.9	54		47	25		~5			
Low-Cl^- ASW	425	8.9	131	9.9	47	206	2.3				5
50 % low-Cl^-	213	4.5	117	5.0	24	78	1.2				5
Low-K^+	434	1.0	496	9.9	47	25	2.3				5
50 % low-K^+	216	1.0	248	5.0	24	13	1.2				5
Na^+-free:low-Ca^{2+}		8.9	427	2.0	47	25		490			
50 % Na^+-free:low-Ca^{2+}		4.5	216	2.0	24	13		245			

* Ca^{2+}-free solutions contain 1 mmol l^{-1} EGTA in both 290 and 460 mosmol kg^{-1}.
All solutions are buffered to pH 7.8.
Content is reduced by half in 460 mosmol kg^{-1} artificial sea water except for Mops and Tris used at 5 mmol l^{-1} as buffers.
Arg^+, arginine; MS^-, methanesulfonate.

encased in a Lucite holder (WPI, Inc.). The reference electrode was a glass pipette filled with 3 mol l^{-1} KCl in 8 % agar and a chlorided silver wire sealed into the end of the pipette with epoxy. The electrodes were connected to a preamplifier (WPI, M-707) with outputs to an oscilloscope, a digital voltmeter and a chart recorder (Grass Instruments 79C). The amount of depolarization following osmotic reduction was calculated as the difference between the resting potential just prior to the reduction in osmolality and the peak of depolarization following osmotic dilution. Where action potentials occurred, the spikes were electronically filtered (half-amplitude frequency=0.1 ms) and the amount of depolarization was calculated from the filtered trace. To estimate the effect of liquid junction potentials at the reference electrode, each solution was tested for its effect with both electrodes in the bath, assuming that the liquid junction potential at the 3 mol l^{-1} KCl-filled microelectrode is negligible. The differences between the electrodes when going from 920 mosmol kg^{-1} to 460 mosmol kg^{-1} in the following solutions were: 1.0±0.4 mV (normal ASW), 2.8±0.9.mV (Na^+-free ASW), 3.6±0.6 mV (Na^+:Ca^{2+}-free ASW), 2.0±2.0 mV (Na^+-free:low-Ca^{2+} ASW), 3.5±1.0 mV [Na^+-free (arginine) ASW], 3.2±0.4 mV (low-Cl^- ASW), 2.2±0.5 mV (low-K^+ ASW), 0.0 mV (Ca^{2+}-free ASW), microelectrode negative. These differences are too small and of the wrong polarity to account for the recorded membrane potential changes. In solutions using sucrose as a NaCl substitute, the differences were larger but again of the wrong polarity to account for the membrane potential changes: 7.8±1.4 mV (Na^+-free:low-Cl^- ASW) and 7.7±0.5 mV (Na^+:Ca^{2+}-free:low-Cl^- ASW), microelectrode negative.

Effect of ouabain on the hypo-osmotic depolarization

After impaling a Parker cell and establishing a stable resting potential in 920 mosmol kg^{-1} ASW, the bath was perfused with 920 mosmol kg^{-1} ASW plus 10^{-4} mol l^{-1} ouabain until a new stable potential was reached. To establish whether the effect of ouabain was maximal at 10^{-4} mol l^{-1}, the bath was then perfused with ASW containing 10^{-3} mol l^{-1} ouabain in three trials. No additional effect was observed by increasing ouabain to 10^{-3} mol l^{-1}; thus, the effect was maximal at 10^{-4} mol l^{-1}. After reaching a stable potential in ouabain, the osmolality was reduced from 920 to 460 mosmol kg^{-1}.

Measuring the change in cell volume

Circumesophageal nerve rings were dissected from animals acclimated to 920 mosmol kg^{-1} for at least 5 days. The abdominal ganglia were separated from the nerve rings and transferred by pipette in a drop of 920 mosmol kg^{-1} ASW to a modified microscope slide that allowed exchange of the solution in a small chamber under the coverslip. Each ganglion was oriented with a Parker cell facing up and the coverslip in contact with the surface of the ganglion. The slide was then mounted on a compound microscope equipped with differential interference contrast (DIC) optics. The cell borders of a Parker cell were resolved using oblique lighting, produced by setting the phase ring slightly off center, in

combination with DIC. The 920 mosmol kg^{-1} ASW in the chamber was then exchanged for 460 mosmol kg^{-1} ASW or 920 mosmol kg^{-1} ASW. The solution in the chamber was replaced with fresh solution every 5 min during the course of the experiment. Video recordings were made using a camera (Dage MTI 67-ML) mounted on the microscope and connected to a video cassette recorder. These recordings were played back and the cross-sectional area of each Parker cell was determined with an image analyser (Bioquant II, R&M Biometrics). The change in cell volume was expressed as a percentage of the initial cross-sectional area of a single optical section of the cell.

Measurement of proline betaine

Circumesophageal nerve rings were dissected from animals acclimated to 920 mosmol kg^{-1}. The nerve rings were then transferred by pipette directly to ASW at 920 or 460 mosmol kg^{-1} in groups of five. At the end of the incubation period (20, 60 or 120 min) each group of nerve rings was transferred to a filter paper (Whatman no. 1). After brief blotting, each group was transferred to a small piece of cellophane, which served as a support for handling and weighing the group of nerve rings. The tissue was then frozen and freeze dried. The dried nerve rings were then weighed to the nearest 10^{-7} g on an ultramicro balance (Mettler, UM-7), homogenized in cold 40 % ethanol, heated just to the boiling point for 10 min, and centrifuged at 20 000 *g* for 20 min (Pierce *et al.* 1984). The pellet was discarded, and the supernatant was freeze dried. The residue was then dissolved in 1 ml of double distilled water and filtered (0.2 mm). The sample was freeze dried again and finally redissolved in 100 ml of 0.040 mol l^{-1} NaH_2PO_3 buffer (pH 4.0).

Proline betaine was measured by high performance liquid chromotography (HPLC) (ALTEX model 334) (Pierce *et al.* 1984) with a reverse-phase column (Microsorb-short one: C-18, 10 cm ODS with 3 mm diameter particles, Rainin Instruments). Proline betaine was detected at 200 nm (Gilson model HM) and chromatograms were analysed with a computer (Shimadzu C-RIA). The mobile phase was 0.040 mol l^{-1} NaH_2PO_4 (pH 4.00). Standards were prepared using proline betaine that had been purified from tissue extracts (Pierce *et al.* 1984).

Measurement of intracellular ions

The intracellular ion content of the ganglia was determined by measuring total tissue ion content and then subtracting the ion content of the extracellular space (ECS) estimated using ^{14}C-labelled polyethylene glycol (M_r 4000) as an ECS marker. For each measurement, three circumesophageal nerve rings from animals acclimated to 920 mosmol kg^{-1} were pooled and incubated for either 20 or 90 min in ASW at 920 or 460 mosmol kg^{-1}. The protocols for incubating the tissues in different experimental media, ECS determination and measurement of tissue Cl^- are described in detail elsewhere (Quinn and Pierce, 1990). For Na^+ and K^+ determination, dried nerve rings were wet-ashed in concentrated HNO_3 overnight. The acid was then dried under a stream of ultrapure N_2, and the residue was dissolved in 2 ml of diluting solution (6 % LiCl in 0.1 mol l^{-1} HNO_3). 1 ml of this

was taken for determination of ECS by liquid scintillation counting. An additional 2 ml of diluting solution was added to the remainder, and $[Na^+]$ or $[K^+]$ was measured by atomic absorption spectrophotometry (Perkin Elmer model 560).

Relationship between bulk loss of Cl^- and the hypo-osmotic depolarization

We tested whether Cl^- loss was dependent on the reduction in osmolality, as is the hypo-osmotic depolarization, or was a response to the reduction in extracellular ions alone. This was done by transferring ganglia from animals acclimated to 920 mosmol kg^{-1} to 920 mosmol kg^{-1} ASW, 460 mosmol kg^{-1} ASW or a solution ionically equivalent to 460 mosmol kg^{-1} ASW with sucrose added to bring the osmolality up to 920 mosmol kg^{-1} (iso-osmotic–hypoionic) for a period of 20 min. Then intracellular Cl^- content was determined as previously described.

Active transport of Na^+ and K^+

Ouabain was used to test for a role of the Na^+/K^+-ATPase in the regulation of the intracellular content of K^+ and Na^+ following hypo-osmotic stress. Ganglia from animals acclimated to 920 mosmol kg^{-1} were transferred to 920 mosmol kg^{-1} or 460 mosmol kg^{-1} ASW with or without ouabain (5×10^{-4} mol l^{-1}) for 20 min before determination of intracellular K^+ content and 90 min before determination of intracellular Na^+ content.

Statistics

Analysis of variance and the Student–Newman–Keuls multiple-range comparison (Sokal and Rohlf, 1969) were used to test for significant differences. Significance was accepted at $P\leq0.05$.

Results

Cell volume regulation

Video images of Parker cells exposed to reduced osmolality initially show an increase in cross-sectional area, indicating an increase in cell volume, followed by a decrease in cross-sectional area, indicating volume recovery (Fig. 1). The accumulated results from a number of such video recordings show that Parker cells swell rapidly during the first 10 min following the reduction in osmolality, and then gradually recover towards the original volume over the next 90 min (Fig. 2). Volume recovery is significant by 60 min.

The hypo-osmotic depolarization

In response to a reduction in osmolality from 920 to 460 mosmol kg^{-1}, Parker cells depolarize for 3 min, spike spontaneously, and then repolarize, taking 20 ± 3 min ($N=9$) to return to the original resting potential (Fig. 3). Thus, recovery of the resting potential requires much less time than cell volume recovery. The hypo-osmotic depolarization does not occur in a solution ionically equivalent to

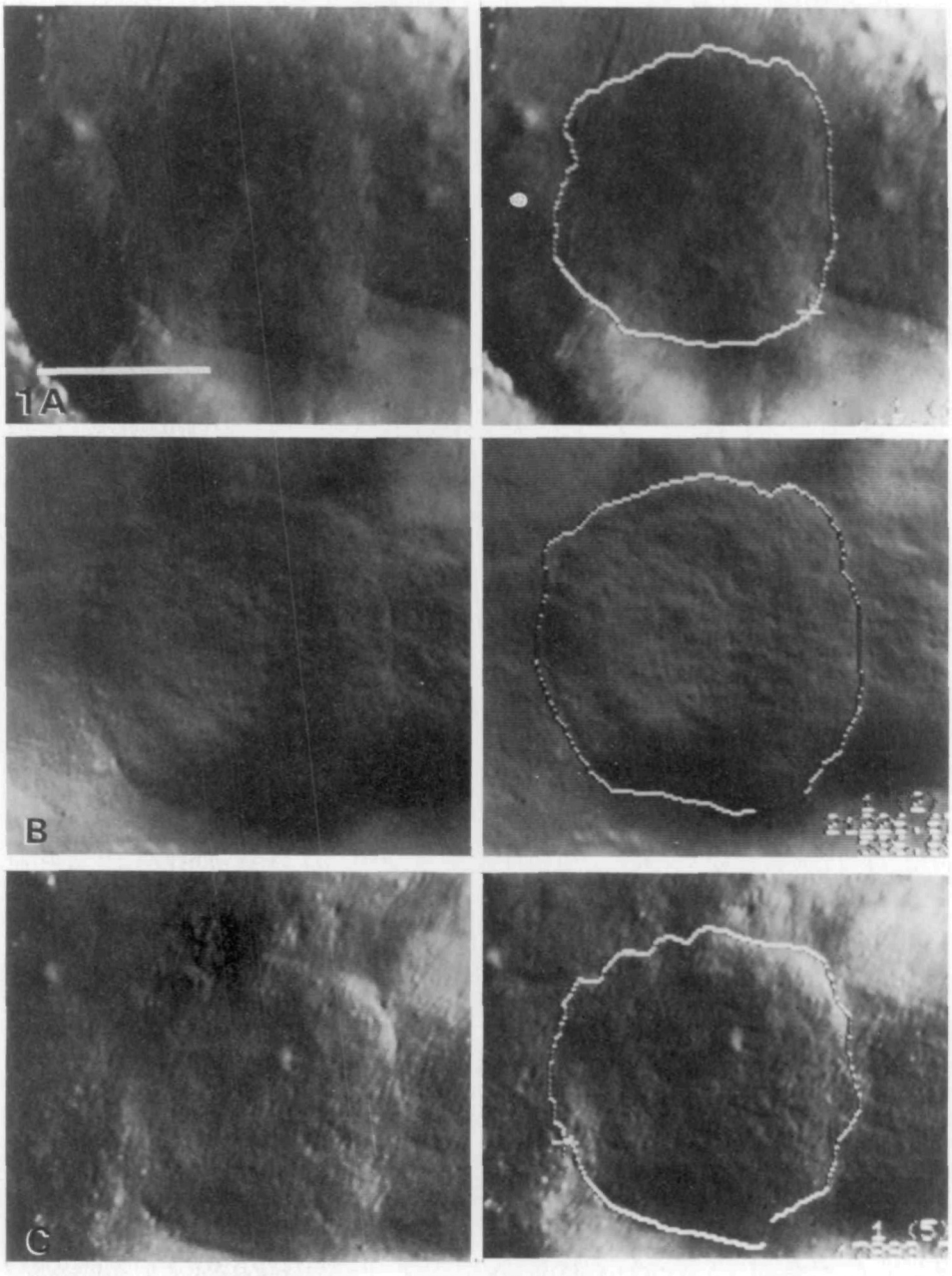

Fig. 1. Photographs of a Parker cell taken from the video monitor of the image analyser. The photographs on the left show the cell at 0 min (A), 20 min (B) and 120 min (C) following transfer from 920 to 460 mosmol kg^{-1} ASW. The photographs on the right show the digitizer tracings of the cell at the same time points. The calculated areas are A, 478 μm^2, B, 536 μm^2, and C, 511 μm^2. Scale bar, 100 μm.

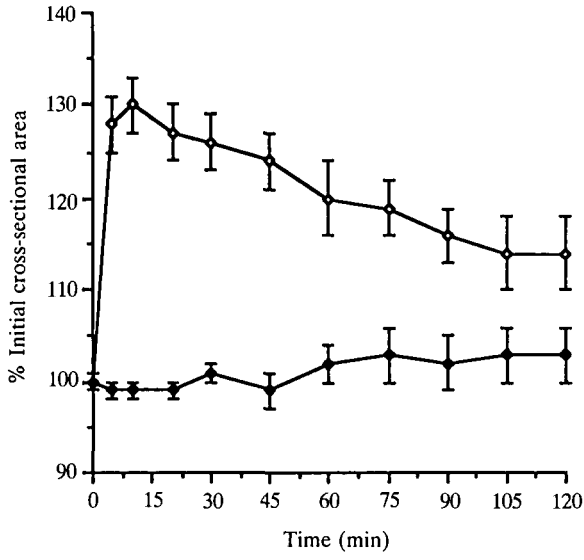

Fig. 2. Change in cell volume of Parker cells after transfer from 920 to 920 mosmol kg^{-1} (◆) or 460 mosmol kg^{-1} (◇) ASW. The change in cell volume is expressed as a percentage of the initial cross-sectional area of an optical section of the cell. Error bars show S.E.M. and N=6 (iso-osmotic) and 9 (hypo-osmotic).

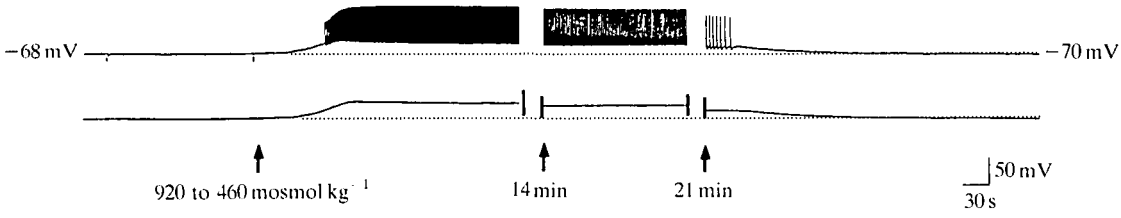

Fig. 3. Hypo-osmotic depolarization in a Parker cell. The upper trace is an intracellular recording of the membrane potential. The lower trace is the same recording with the spikes electronically filtered (see text). Dotted lines are at −68 mV.

460 mosmol kg^{-1} ASW while the osmolality is maintained at 920 mosmol kg^{-1} (sucrose) (Fig. 4). Furthermore, cells transferred from sucrose-substituted ASW at 920 mosmol kg^{-1} to ASW at 460 mosmol kg^{-1} depolarize immediately (44±2 mV, N=3) (Fig. 4).

Manipulating the level of K^+ or Cl^- in the ASW had no significant effect on the hypo-osmotic depolarization, while substituting $Tris^+$ or arginine for Na^+ significantly reduced the hypo-osmotic depolarization (Table 2). Although both Na^+ substitutes significantly reduced the depolarization, $Tris^+$ was more effective than arginine. However, even in Na^+-free $Tris^+$, 10 mV of the depolarization was still unaccounted for. The hypo-osmotic depolarization remaining in Na^+-free solution was not affected by reducing or removing Ca^{2+} (Table 3).

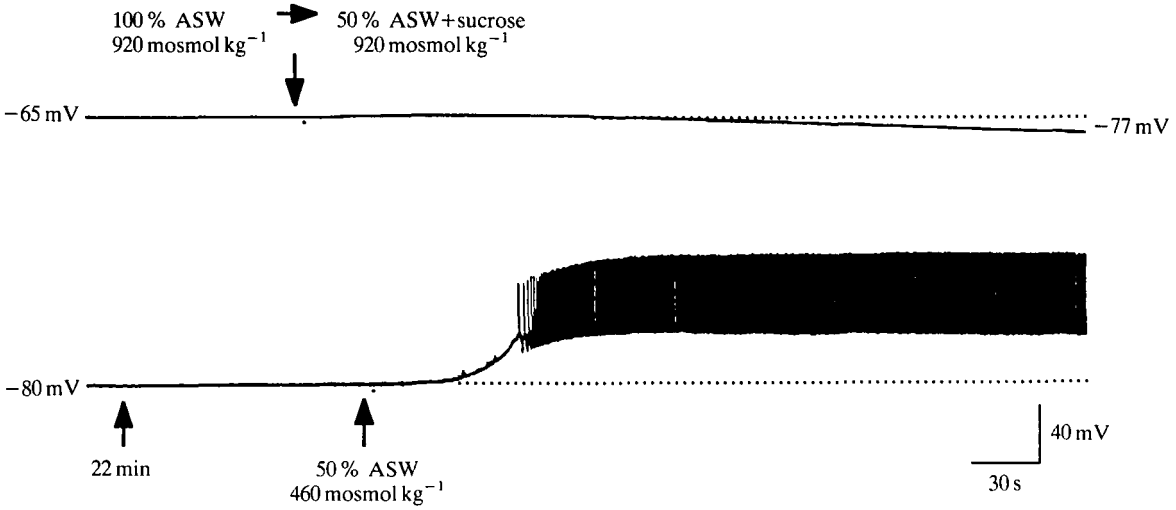

Fig. 4. Unfiltered intracellular recording from a Parker cell showing the effect of iso-osmotic *vs* hypo-osmotic salinity reduction. In the upper trace, the ionic concentration is reduced by 50 % while osmolality is kept iso-osmotic with 100 % ASW by adding sucrose. The lower trace is from the same cell at a later time. After 22 min in 50 % ASW+sucrose the osmolality is finally reduced by superfusion with 50 % ASW. Dotted lines are at −65 mV (upper trace) and −80 mV (lower trace).

Table 2. *Hypo-osmotic depolarization in normal ASW* vs *Cl^--, K^+- or Na^+-substituted ASW*

Treatment (920 to 460 mosmol kg^{-1})	Mean depolarization±S.E.M. (mV)	*N*
Normal ASW	31±1	10
Low-Cl^- ASW	36±3	6
Low-K^+ ASW	33±3	5
Na^+-free (arginine) ASW	21±3	4
Na^+-free ($Tris^+$) ASW	10±1	11

See Table 1 for solution compositions.

The hypo-osmotic depolarization remaining in the Na^+-free solution was significantly increased by reducing Cl^- concentration, by replacing NaCl with either sucrose or trismethanesulfonate (TrisMS) (Table 3). The Na^+-free hypo-osmotic depolarization was also increased by quinidine (Table 3). Quinidine had no effect on the membrane potential in iso-osmotic Na^+-free solution.

Incubation of the Parker cells in 920 mosmol kg^{-1} ASW containing 10^{-4} mol l^{-1} (or 10^{-3} mol l^{-1}) ouabain produced a small depolarization, 6±1 mV (N=6) above resting potential (Fig. 5). Subsequent osmotic reduction from 920 to 460

Table 3. *Hypo-osmotic depolarization in Na^+-free ASW: effect of Ca^{2+}-free, low-Cl^- and quinidine treatments*

Treatment (920 to 460 mosmol kg^{-1})	Mean depolarization±S.E.M. (mV)	*N*
Na^+-free (TrisHCl)	10±1	11
Na^+-free:low-Ca^{2+}	12±4	3
Na^+:Ca^{2+}-free	11±2	14
Na^+-free:low-Cl^- (sucrose)	29±4	5
Na^+-free:low-Cl^- (TrisMS)	23±2	4
Na^+-free (TrisHCl)+1 mmol l^{-1} quinidine	25±2	5

See Table 1 for solution compositions.

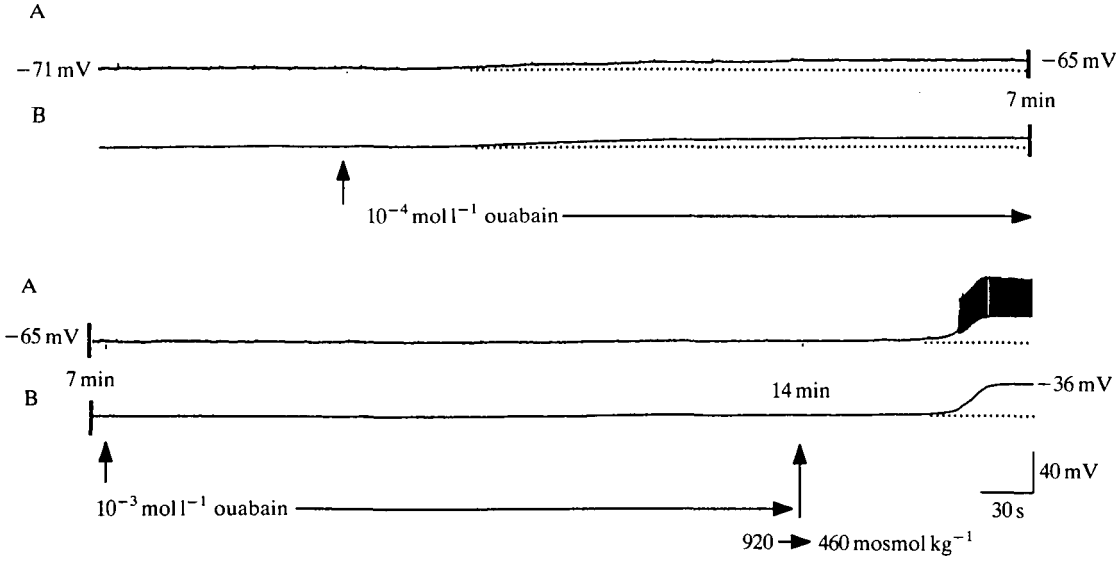

Fig. 5. Intracellular recording from a Parker cell showing the effect of ouabain on the membrane potential and the hypo-osmotic depolarization. A and B are simultaneous recordings; B is filtered. The lower pair of traces is a continuation of the upper pair. Dotted lines for the upper pair of traces are at −71 mV, the resting potential of the cell before exposure to ouabain. Dotted lines for the lower pair of traces are at −65 mV, the resting potential after treatment with 10^{-4} mol l^{-1} ouabain. Note that increasing the concentration of ouabain to 10^{-3} mol l^{-1} did not cause further depolarization, whereas reducing the osmolality did.

mosmol kg^{-1} produced an additional depolarization of 28±1 mV (N=6), which is not significantly different from the hypo-osmotic depolarization observed without pre-treatment in ouabain (31±1 mV, N=13).

Intracellular osmolytes

The main organic osmolyte in the ganglia, proline betaine, leaves the tissue throughout the 2 h period following hypo-osmotic stress (Fig. 6A), but not significantly so until after the 20 min sampling period. After 2 h, proline betaine content is decreased from 784 μmol g^{-1} dry mass in iso-osmotic controls to 404 μmol g^{-1} dry mass in hypo-osmotically exposed ganglia.

Intracellular Na^+ content declines in hypo-osmotically treated ganglia compared with iso-osmotic controls, but not significantly until after the 20 min sampling period (Fig. 6B). By the 90 min sample, intracellular Na^+ is reduced from 307 μmol g^{-1} dry mass in iso-osmotic controls to 250 μmol g^{-1} dry mass in hypo-osmotically exposed ganglia. In ganglia exposed to ouabain (5×10^{-4} mol l^{-1}) for 90 min, intracellular Na^+ content increases significantly in both iso-osmotic and hypo-osmotic media by 384 and 460 μmol g^{-1} dry mass, respectively, and there is no significant difference in intracellular Na^+ concentration between iso-osmotically and hypo-osmotically treated ganglia (Fig. 6C). Thus, the loss of Na^+ that follows hypo-osmotic stress is inhibited by ouabain.

There is no significant difference in intracellular K^+ content between hypo-osmotically treated ganglia and iso-osmotic controls during the 90 min test period. Thus, unlike the other osmolytes, intracellular K^+ is conserved following hypo-osmotic stress. Ouabain (5×10^{-4} mol l^{-1} for 20 min) causes a significant decrease in intracellular K^+ concentration in both iso-osmotic and hypo-osmotic media by 114 and 172 μmol g^{-1} dry mass, respectively; and intracellular K^+ concentration is significantly lower in hypo-osmotically treated ganglia in the presence of ouabain (Fig. 6D). Thus, the ability of the cells to conserve K^+ following hypo-osmotic stress is inhibited by ouabain.

Intracellular Cl^- content is reduced significantly by 20 min of hypo-osmotic stress. The majority of intracellular Cl^- loss occurs during the first 20 min (Quinn and Pierce, 1990). The intracellular Cl^- content is also reduced significantly from 330 μmol g^{-1} dry mass in iso-osmotic controls to 199 μmol g^{-1} dry mass in iso-osmotic–hypoionically treated ganglia (Fig. 6E). Further, there is no significant difference in intracellular Cl^- content between hypo-osmotic and iso-osmotic–hypoionically treated ganglia (Fig. 6E). Thus, the reduction in osmolality is not required for the loss of Cl^- from the cells, only the accompanying decrease in $[Cl^-]_o$.

Discussion

It has been suggested that membrane potential changes during osmotic stress might accompany cell volume recovery (Gilles, 1987). Such a theory must presume either that the time course of osmolyte loss is contolled by the membrane potential changes or that osmolyte loss by electrogenic pathways follows the full time course of volume regulation. A portion of the solute lost during hypo-osmotic stress consists of organic osmolytes, such as free amino acids or quaternary ammonium compounds, which have no net charge. If these osmolytes were to leave the cells

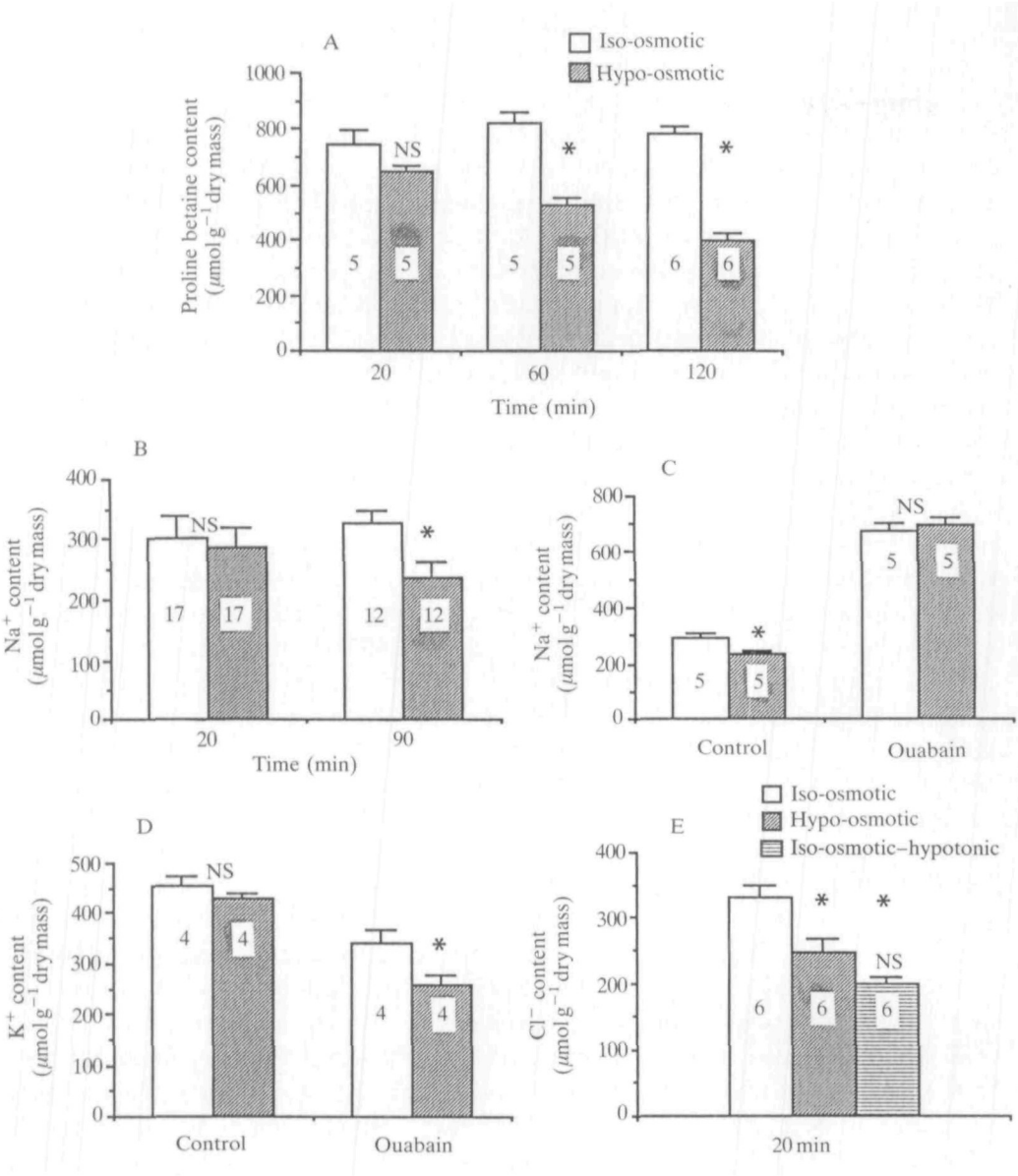

Fig. 6. Intracellular solute content in *Elysia chlorotica* circumesophageal ganglia following transfer from 920 mosmol kg^{-1} to 920 mosmol kg^{-1} (iso-osmotic) or to 460 mosmol kg^{-1} (hypo-osmotic) ASW. (A) Proline betaine content. (B) Na^+ content. (C) Effect of ouabain on Na^+ content. (D) Effect of ouabain on K^+ content. (E) Cl^- content. In this experiment, a third group of ganglia was transferred to a 920 mosmol kg^{-1} solution made by adding sucrose to 460 mosmol kg^{-1} ASW (iso-osmotic–hypoionic). Error bars show S.E.M. An asterisk indicates a significant difference from the iso-osmotic control, $P \leq 0.05$; NS, not significant. Values of N are given in the columns. There is no significant difference between the chloride contents of the hypo-osmotically and iso-osmotic–hypoionically treated ganglia.

by electrically neutral pathways, at least a portion of cell volume regulation would occur in the absence of membrane potential changes. The loss of these organic osmolytes often occurs more slowly than the loss of inorganic ions and accounts for most of the cell's capacity to recover volume (Pierce, 1982). Thus, even if inorganic ions leave the cells by conductive pathways, volume recovery may occur over a time course that is very different from that of the hypo-osmotic depolarization. Indeed, the depolarization in Parker cells occurs before the peak in cell volume (which is probably limited by ion loss), and recovery of the resting potential occurs before volume recovery, which is produced by proline betaine loss.

The results of the ion substitution experiments indicate that Na^+ permeability accounts for most of the hypo-osmotic depolarization in Parker cells. This could occur in two ways, either by an increased relative permeability to Na^+ or by inhibition of the Na^+/K^+-ATPase in the presence of an already existing and relatively large Na^+ leak. Since the depolarization was not altered by ouabain, the results suggest that increased relative permeability to Na^+ is the case. The activation of a permeability pathway favoring an inward movement of Na^+ is of no osmotic benefit to a cell that needs to lose solute in order to regulate volume. However, the influx of Na^+ by this pathway may be very small and rapidly opposed by the Na^+/K^+-ATPase. Indeed intracellular Na^+ content does not increase during hypo-osmotic exposure.

The gradient for Na^+ is strongly inward and the observed reduction in intracellular Na^+ concentration following hypo-osmotic stress does not occur in the absence of active transport. Similarly, the reduction in intracellular Na^+ concentration in *Mercierella enigmatica* axons following salinity reduction is ouabain-sensitive (Benson and Treherne, 1978), but in the mammalian kidney cortex (Gilles *et al.* 1983) and *Limulus polyphemus* heart (Warren, 1982) it is not. In most cell types, aniso-osmotic volume recovery is not inhibited by ouabain, indicating that the Na^+/K^+-ATPase is not required for volume recovery (Rorive and Gilles, 1979). The present data indicate that the Na^+/K^+-ATPase can contribute a part of the total osmolyte loss.

There are at least two explanations for the hypo-osmotic depolarization that remains in the absence of Na^+. First, the differences between our results obtained with arginine and $Tris^+$ suggest that $Tris^+$ may not be completely impermeant, thus causing some depolarization. The permeability of Na^+ channels to organic cations varies in different tissues and even $Tris^+$ can be highly permeant (Edwards, 1982). Second, it is possible that other ions contribute to the depolarization. Our results rule out Ca^{2+} in that regard, since reducing or removing Ca^{2+} in Na^+-free ASW does not affect the level of depolarization. However, the increased hypo-osmotic depolarization that occurs when Cl^- concentration is reduced in Na^+-free ASW indicates that an increased relative permeability to Cl^- may account for the remaining depolarization. This Cl^- component of the hypo-osmotic depolarization could also represent an osmotically activated pathway for Cl^- loss from the cells.

Most of the Cl^- loss from *Elysia* cells occurs during the first 20 min after transfer

to hypo-osmotic ASW. Thus, unlike that for the other osmolytes, the time course of Cl^- loss correlates with that of the hypo-osmotic depolarization (Quinn and Pierce, 1990). However, an equal loss of Cl^- occurs from a reduction of ions alone in iso-osmotic media (iso-osmotic–hypoionic), a condition in which a hyperpolarization rather than a depolarization occurs. This result indicates that an osmotically activated pathway is not required for a substantial loss of Cl^- from the cells and that bulk loss of Cl^- and the hypo-osmotic depolarization are unrelated.

Since the hypo-osmotic stress causes cellular swelling, it is possible that a stretch-activated Na^+ channel is responsible for the depolarization. In striking similarity to our results from Parker cells, a stretch-activated channel isolated from opossum kidney cells conducts an inward cation current, but in the absence of extracellular Na^+ carries an outward Cl^- current (Ubl *et al.* 1988). The activation of such a channel would result in membrane depolarization in the presence or absence of Na^+, given an outward gradient for Cl^-. A channel with similar properties may be responsible for the hypo-osmotic depolarization in Parker cells. Such a channel may be utilized in neuronal functions such as the electromechanical transduction described in axons and stretch receptors (Sachs, 1986).

Volume-activated K^+ conductances in Ehrlich ascites tumor cells, human peripheral blood lymphocytes and pancreatic β-cells are inhibited by quinine (Hoffmann *et al.* 1984; Sarkadi *et al.* 1984; Marcstrom *et al.* 1990). Thus, the increase in the Cl^- component induced by quinidine could result from inhibition of a volume-activated K^+ conductance, possibly a means for K^+ to leave the cells and serve as a counterion for loss of Cl^-. However, intracellular K^+ content is unchanged in *Elysia* ganglia following hypo-osmotic stress. Thus, K^+ does not contribute to cell volume regulation. This result contrasts with those from a number of other cell types in which hypo-osmotic stress activates efflux of K^+ along with Cl^-, either by separate conductances or by electrically neutral cotransport or exchange mechanisms (Eveloff and Warnoch, 1987; Hoffmann and Simonsen, 1989). Intracellular K^+ concentration is also reduced in axons from the euryhaline osmoconformers *Mytilus* (Willmer, 1978*a*) and *Mercierella* (Benson and Treherne, 1978) following salinity reduction, but not in proportion to the reduction in extracellular K^+ concentration. Treherne (1980) concluded that these molluscan neurons require a certain amount of intracellular K^+ for maintenance of their excitability characteristics at low salinity. Failure to reduce $[K^+]_i$ in proportion to $[K^+]_o$ increases the K^+ ratio across the membrane at low salinities, resulting in a hyperpolarized resting potential. The increased ratio compensates for the reduction in action potential overshoot produced by the reduction in extracellular Na^+ concentration, thus maintaining the amplitude and rate of rise of the action potential (Treherne, 1980). A hyperpolarized resting potential also occurs in Parker cells after acclimation to low salinity (Parker and Pierce, 1985). Thus, *Elysia* neurons may conserve K^+ in order to maintain excitability during exposure to very dilute sea water.

In *Elysia* ganglia, the effect of ouabain on intracellular K^+ concentration in hypo-osmotic *vs* iso-osmotic media indicates that activity of the Na^+/K^+-ATPase

is required for the maintenance of intracellular K^+ concentration during low-salinity stress. Since extracellular K^+ concentration was reduced by half when *Elysia* ganglia were transferred from 920 to 460 mosmol kg^{-1}, the complete conservation of intracellular K^+ content would require either a reduced K^+ leak or increased active transport. A 76% increase in Na^+ pump sites, measured by binding of [^{3}H]ouabain, occurs in *Mytilus* axons after acclimation to 25% sea water from 100% sea water, indicating that active transport by the Na^+/K^+-ATPase increases at low salinity (Willmer, 1978*c*). A similar response may account for the conservation of intracellular K^+ in *Elysia* ganglia during low-salinity stress.

In conclusion, we have observed a depolarization in neurons which occurs only in response to a reduction in osmolality and not to an iso-osmotic reduction in ions. The electrophysiological evidence indicates that this depolarization is primarily the result of an increased relative permeability to Na^+ and is, therefore, not the result of bulk loss of intracellular ions. The measurements of intracellular solutes support that conclusion, since the only osmolyte that is reduced coincident with the depolarization, Cl^-, is equally reduced by an iso-osmotic reduction in ions, a manipulation that produces hyperpolarization rather than depolarization. Thus, the hypo-osmotic depolarization in neurons has no apparent relationship to cell volume regulation. Possibly, the hypo-osmotic depolarization results from some other neuronal property, such as transduction of mechanical stimuli (osmotically induced stretch) into electrical signals.

This work was supported in part by NSF grant no. DCB-8710067.

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