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A CHLORIDE CURRENT ASSOCIATED WITH SWELLING OF CULTURED CHICK HEART CELLS

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SUMMARY

1. Cultured chick heart cells challenged by hyposmotic stress underwent regulatory volume decrease (RVD) that was attenuated by prior depletion of intracellular chloride.

2. During hyposmotic swelling, cell aggregates experienced an initial increase in spontaneous contractile activity followed by eventual quiescence. Conventional microelectrode studies revealed an underlying increase in spontaneous electrical activity, followed by a sustained depolarization beyond threshold.

3. Whole-cell patch clamp studies, with K^+ currents blocked, indicated that exposure of cells to hyposmotic solution (NaCl reduction) resulted in a rapid osmotic swelling followed by a substantial increase in whole-cell conductance which persisted for the duration of hyposmotic exposure and was almost completely reversed on return to isosmotic bath solution.

4. For a variety of Cl^- concentrations, the reversal potentials (E_{rev}) of the measured swelling-activated current closely followed the calculated Cl^- equilibrium potential (E_{Cl}) with a linear regression slope of 0·82. When estimated by the Nernst equation, the relationship between E_{rev} and the $[Cl^-]_i/[Cl^-]_o$ ratio fitted well with a slope of 51 mV per decade change in the concentration ratio, consistent with a Cl^--selective conductance.

5. The permeability ratios of this swelling-activated conductance to chloride, methanesulphonate (MSA) and aspartate (Asp) were calculated as $P_{Cl}:P_{MSA}:P_{ASP} = 1:0{\cdot}36:0{\cdot}02$, with the ion selectivity sequence of $Cl^- > MSA^- \gg Asp^-$, which suggests the swelling-activated conductance is slightly permeable to other anions.

6. Application of a Cl^- channel blocker, diphenylamine-2-carboxylate (DPC, 200 μM), substantially suppressed the swelling-activated current without shifting the E_{rev} of this current. The effect of DPC was independent of membrane potential.

7. This evidence demonstrates that hyposmotic swelling of cultured chick heart cells activates a channel-mediated Cl^- conductance which may be associated with the integrated response of volume-regulatory mechanisms.

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INTRODUCTION

Volume-regulatory mechanisms play a critical role in maintaining structural integrity and proper functioning of living cells (Hoffmann & Simonsen, 1989; Sarkadi & Parker, 1991). Like many other cell types, cardiac cells are known to regulate their volume in response to osmotic swelling (Page & Storm, 1966; Houser & Freeman, 1979; Desai, Jacob, Lieberman & Rosenthal, 1986; Roos, 1986; Drewnowska & Baumgarten, 1991; Rasmusson, Davis & Lieberman, 1993), a process usually referred to as the regulatory volume decrease (RVD). During RVD, cytoplasmic solutes and osmotically obliged water are extruded and cell volume returns towards the control value. Although the net loss of inorganic solutes in cardiac cells during RVD have usually been attributed to the net efflux of K^+ and Cl^- (Jennings, Reimer & Steenbergen, 1986; Rasmusson *et al.* 1993), the exact membrane transport mechanisms responsible for these volume-regulatory ion movements in cardiac cells have not been identified. In cultured chick heart cells, the RVD associated with hyposmotic swelling has been clearly demonstrated to be mediated in part by the efflux of amino acids, with the remainder postulated to be mediated by extrusion of the inorganic solutes, although the specific pathways for inorganic solute loss have not been identified (Rasmusson *et al.* 1993).

The cardiac action potential is associated with permeability changes of the cell membrane which, in the absence of counterbalancing active transport pathways to maintain ion homeostasis, could alter the intracellular milieu and ultimately affect cell volume and electrical activity. Even though cardiac cells do not encounter substantial changes in the osmolarity of extracellular fluid under physiological conditions, cardiac cell volume homeostasis can be disturbed under patho-physiological conditions. Cardiac cell swelling during myocardial ischaemia and reperfusion provides an important indication of progressing cell injury (Steenbergen, Hill & Jennings, 1985; Jennings *et al.* 1986; Ganote & Vander Heide, 1988). Although the origins of cell swelling may vary considerably, the net result is an increase in the intracellular content of osmotically active solutes such that the osmotic gradient across the cell membrane causes water influx and cell swelling.

Three major ion transport mechanisms have been postulated to mediate RVD in other cell types, namely cation coupled cotransport (either K^+–Cl^- or Na^+–K^+–$2Cl^-$), K^+–H^+ exchange functionally coupled to Cl^-–HCO_3^- exchange or electrochemically coupled independent K^+ and Cl^- electrodiffusive pathways (see recent reviews by Grinstein, Rothstein, Sarkadi & Gelfand, 1984; Hoffmann & Simonsen, 1989; Sarkadi & Parker, 1991). In cardiac cells, activation of electrodiffusive pathways associated with RVD could influence the electrophysiological properties of the myocardium. K^+ conductance is dominant near the resting potential and so the main requirement to activate a volume regulatory electrodiffusive KCl efflux would involve an increase in Cl^- conductance, which is very low at rest in cultured chick heart cells (Piwnica-Worms, Jacob, Horres & Lieberman, 1983) and in unstimulated mammalian cardiac cells (Bahinski, Nairn, Greengard & Gadsby, 1989; Harvey, Clark & Hume, 1990). Activation of a Cl^- conductance during RVD, however, will certainly alter cardiac electrical activity, particularly with respect to repolarization and generation of the diastolic depolarization underlying pacemaker activity. A

swelling-induced Cl^--sensitive current has recently been reported in cardiac cells (Sorota, 1992; Tseng, 1992; Hagiwara, Masuda, Shoda & Irisawa, 1992), although its contribution to cardiac cell volume regulation has not been established.

The results reported in the present study provide direct evidence that osmotic swelling in cultured chick heart cells is tightly associated with a substantial increase in membrane conductance. Activation of a Cl^- channel during cell swelling contributes to this increase in membrane conductance, and is consistent with the observed membrane depolarization during RVD. These results suggest that the loss of inorganic solutes in cultured chick heart cells during RVD is associated with the activation of a Cl^- conductive pathway. Preliminary results of this study have been presented previously in abstract form (Zhang, Rasmusson, Hall & Lieberman, 1992*a*, *b*).

METHODS

Cell preparation

Eleven-day-old chick embryos were extirpated aseptically from the eggs and immediately decapitated; the hearts were rapidly removed and minced. Single heart cells were isolated by enzymatic dissociation in the absence of antibiotics, as previously described (Jacob, Lieberman & Liu, 1987). The myocyte-enriched supernatant was incubated overnight at 37 °C to produce single spherical myocytes (15–20 μm diameter) for the whole-cell patch clamp studies, or for 3 days to produce either small spherical aggregates (Rasmusson *et al.* 1993) or polystrand preparations (Liu, Piwnica-Worms & Lieberman, 1990) for volume and microelectrode studies, as indicated in the text.

Cell volume perturbation and measurement

Cell volume perturbation was accomplished by perfusing cells with hyposmotic external solutions. Unless otherwise indicated, hyposmotic solutions contained 50% NaCl with a reduction of osmolarity from ~ 290 mosmol/l (isosmotic) to ~ 164 mosmol/l.

Volumes of single cells or cell aggregates were calculated from measured diameter changes as described previously; these morphological measurements have been demonstrated to correlate well with measured changes in cell water (Rasmusson *et al.* 1993). All volumes were normalized to their control values before volume perturbation.

Contractile measurement

Contractile activity of chick heart cells was measured from spontaneously active polystrand preparations, using an edge detecting device (Steadman, Moore, Spitzer & Bridge, 1988). To provide a strong contrast edge, the polystrand preparations were decorated with latex beads ~ 1 h before experiments (Steadman *et al.* 1988). Definition of the video signal was improved by use of a video enhancer (Archer 15-1270, Radio Shack, Fort Worth, TX, USA) whose output was fed into the edge detecting device to record the contraction of preparations.

Electrophysiological studies

Transmembrane potential (E_m) was recorded from spontaneously active polystrand preparations, using conventional glass microelectrodes filled with 3 M KCl (8–10 MΩ). Whole-cell currents were measured using the whole-cell patch clamp technique developed by Hamill, Marty, Neher, Sakmann & Sigworth (1981). Patch pipettes were fabricated from borosilicate glass capillary tubing (7052, Garner Glass Co., Claremont, CA, USA), coated with Sylgard 184 (Dow-Corning, Midland, MI, USA) and fire polished; they had tip resistances of 5–10 MΩ when filled with pipette solution. A high resistance seal ($\geqslant$ 10 GΩ) was formed by applying gentle negative pressure. The patch was ruptured by a brief suction pulse to establish the whole-cell configuration. Electrode solution was maintained at atmospheric pressure throughout the experiments.

Current records were obtained using a Dagan 8900 patch clamp amplifier (Dagan Corp., Minneapolis, MN, USA). Currents were low-pass filtered at 2 kHz by a four-pole Butterworth filter

(APVL/H, A. P. Circuit Corp., New York, NY, USA), and either directly digitized by an IBM PC/AT computer using an Axolab-1 acquisition system (Axon Instruments Inc., Burlingame, CA, USA) or stored on videotape (SL-2700 video recorder, Sony, Tokyo, Japan) using a PCM converter (PCM-501ES, Sony) for later analysis. Voltage clamp protocols were generated and data were acquired and analysed using version 5.5 of pCLAMP software (Axon Instruments Inc.). Possible changes in junction potential between the pipette solution and the external solution were corrected through a bath reference amplifier (Model 8950, Dagan Corp.) by placing a 3 M KCl-filled reference microelectrode close to the cell preparation.

Whole-cell currents were elicited using either voltage step or ramp protocols from a holding potential of −40 mV. Voltage steps were applied once every 2 s, and voltage ramps were applied at a rate of −0·5 V/s every 10–20 s. For comparison, a standard current–voltage (I–V) relationship was determined by voltage step protocol. The distributed capacitance was compensated after the gigaohm seal was achieved. Cell membrane capacitance was estimated by integrating the transient current response to a 5 mV hyperpolarizing step and dividing by that voltage step. The average cell capacitance was calculated to be 7·9 ± 1·5 pF (mean ± S.D., $n = 30$). Cell capacitance and series resistance were not compensated during the experiments. All whole-cell currents measured were normalized to cell membrane capacitance (pA/pF).

Ion content measurements

Methods for measuring cellular Na^+ and K^+ content have been described in detail (Murphy, Aiton, Horres & Lieberman, 1983). Briefly, cultured cells grown in 35 mm dishes were incubated in test solutions for 20 min. Extracellular cations were removed by rinsing the cells with 10 ml of ice-cold buffered choline chloride solution. Na^+ and K^+ were extracted from the cells with 0·75 N HNO_3 and analysed by atomic absorption spectroscopy. Protein was extracted from each dish with 0·1 N NaOH and assayed according to Lowry, Rosenbrough, Farr & Randall (1951). Ion contents were expressed as nanomoles per milligram of cell protein.

Solutions

The control external solution was a Hepes–Tris-buffered salt solution (HTBSS) of the following composition (mM): 142·6 NaCl, 5·4 KCl, 0·8 NaH_2PO_4, 0·8 $MgSO_4$, 2·0 $CaCl_2$, 10 Hepes–Tris, 5·6 dextrose, 0·1 % bovine serum albumin, adjusted to pH 7·4. Solutions were rendered hyposmotic by reduction of the NaCl concentration. Throughout the text and figure legends, NaCl concentration is expressed as a percentage of control (see Table 1). The osmolarity of all solutions was measured with a vapour pressure osmometer (Model 5100B, Wescor Inc., Logan, UT, USA). In electrophysiological experiments, sodium methanesulphonate (NaMSA) was used as a partial substitute for NaCl in isosmotic solution, such that $[Cl^-]_o$ remained constant throughout. The solution used to block K^+ currents was prepared by adding 1 mM $BaCl_2$ and replacing NaH_2PO_4 and $MgSO_4$ with equimolar concentrations of NaCl and $MgCl_2$, respectively. External chloride concentration was altered by replacing NaCl with an equimolar concentration of NaMSA or an equiosmolar concentration of sucrose, as indicated in the text. In Cl^--free solution, all chloride salts were replaced with methanesulphonate salts. In Na^+-free solution, Na^+ was substituted with equimolar *N*-methyl-D-glucamine (NMDG). The Cl^- channel blocker diphenylamine-2-carboxylate (DPC; Aldrich, Milwaukee, WI, USA) was dissolved in dimethyl sulphoxide (DMSO; Aldrich). The final concentration of DMSO (0·1 %) did not affect the electrical or contractile activity of cultured chick heart cells.

The control pipette solution contained (mM): 30 KCl, 110 potassium aspartate, 2 $MgCl_2$, 0·5 $CaCl_2$, 1·0 EGTA, 10 Hepes–Tris, 5·0 Na_2ATP, adjusted to pH 7·2. Potassium-free solution was prepared by substitution of K^+ with Cs^+. Variation of $[Cl^-]_i$ was made by replacing aspartate with an equimolar concentration of Cl^-.

All the experiments were performed at 37 °C.

Data analysis and statistics

Unless otherwise indicated, all data were presented as digitized recordings or, in the case of a series of measurements, as arithmetic means ± S.E.M. (n), where n represents the number of experiments. The I–V curves for the voltage step clamp were obtained by a single exponential fit

to the data assessed by a least-squares test. All reversal potentials of current–voltage relationships were measured from their single exponential fitted curves. Statistical analysis was made by Student's t test for paired or unpaired data and a significant difference was assumed at $P<0{\cdot}05$.

RESULTS

Loss of inorganic solutes during RVD in hyposmotic solutions

When exposed to hyposmotic bath solution, chick heart cell aggregates swell within the first few minutes and then undergo a regulatory volume decrease (RVD) toward their original size (Rasmusson *et al.* 1993). Although a substantial portion of the RVD was shown to be mediated by efflux of amino acids, the loss of inorganic solutes was also thought to play an important role during RVD. To examine the changes in intracellular inorganic solutes in response to a hyposmotic challenge, cellular Na^+ and K^+ contents were measured during volume perturbation. Table 1 shows the relative values of intracellular Na^+ and K^+ contents in control, 50 and 25% NaCl solutions. The increasing loss of inorganic ions is clearly associated with the degree of cell swelling and was largely reversed on return to isosmotic solution, as shown in Table 2. Thus, a volume-sensitive mechanism exists in cultured chick heart cells to adjust the inorganic ion content in response to changes in the osmotic environment. We examined the importance of intracellular chloride to RVD (Fig. 1). Chick heart cell aggregates were first perfused for 20 min in an isosmotic solution in which all Cl^- had been substituted with impermeant methanesulphonate (MSA) to deplete intracellular Cl^- (Liu, Jacob, Piwnica-Worms & Lieberman, 1987). This manipulation caused cells to shrink due to the depletion of internal Cl^- (Rasmusson *et al.* 1993). When the aggregates were exposed to a hyposmotic Cl^--free solution (50% of NaMSA in the isosmotic control solution), the RVD in response to swelling was attenuated and the time course was markedly slowed. At the end of 20 min perfusion in hyposmotic Cl^--free solution, the relative volume of cell aggregates stayed high ($1{\cdot}36 \pm 0{\cdot}04$, $n = 5$), in comparison with those under Cl^--containing conditions ($1{\cdot}20 \pm 0{\cdot}02$, $n = 7$; $P < 0{\cdot}005$). These results suggest the involvement of Cl^--dependent mechanisms in the RVD.

Changes in contractile and electrical activities during RVD

Multicellular preparations of cultured chick heart cells show spontaneous rhythmic activity. Exposure to hyposmotic solutions (50% NaCl) consistently produced a rapid acceleration of contractile activity leading to quiescence within a few minutes (Fig. 2). Normal contractile activity recovered after a few minutes reperfusion in isosmotic solution.

Changes in contractile rate are a result of changes in the electrophysiological properties. We used conventional high-resistance microelectrodes during the hyposmotically activated RVD to monitor changes in membrane potential associated with the observed changes in contractile behaviour. Figure 3*A* shows a continuous record of the transmembrane potential from a spontaneously active polystrand preparation. Cell swelling induced by a 25% NaCl solution was associated with an increase in the rate of spontaneous electrical activity and eventual quiescence, as the membrane depolarized beyond its excitation threshold. The similarity between

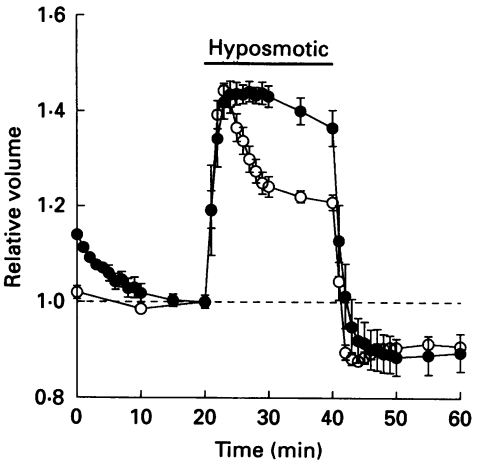

Fig. 1. Volume-regulatory response of chick heart cell aggregates in the presence and absence of chloride. Cl^--free solution was prepared by substitution of chloride with methanesulphonate (MSA) in the bath solution. Cell aggregates were initially perfused with isosmotic solution for 20 min, followed by 20 min of perfusion with hyposmotic solution (50% NaCl for control (○) and 50% NaMSA for Cl^--free solution (●)). Cell aggregates were reperfused with isosmotic solution after hyposmotic swelling. For each point $n = 5$.

TABLE 1. Na^+ and K^+ contents of cardiac cells following exposure to hyposmotic solutions

$[NaCl]_o$ (%)	Osmolarity (mosmol/l)	Na^+	K^+	Total	
		(nmol/mg protein)			n
100	293	115±5	1196±26	1310±25	5
50	164	102±11	1135±44	1237±55	5
25	98	70±9*	726±36*	796±33*	5

Cell aggregates in each group were initially perfused in isosmotic control solution for 20 min, and then switched to solutions with different osmolarity as indicated in the table. Total Na^+ and K^+ contents were measured at the end of a 20 min exposure to the above solutions. All solutions were Hepes–Tris-buffered solutions. Osmolarity was adjusted by omission of NaCl in control isosmotic solution (100% $[NaCl]_o$ contained 142·6 mM NaCl). n, the number of experiments; * statistically different from control, $P < 0.05$ using Student's unpaired t test.

TABLE 2. Na^+ and K^+ contents in control solution after 20 min recovery from RVD

$[NaCl]_o$ (%)	Osmolarity (mosmol/l)	Na^+	K^+	Total	
		(nmol/mg protein)			n
100	293	133±50	1125±28	1258±29	4
50	164	157±20	1156±22	1313±41	4
25	98	174±10*	990±22*	1163±28*	4

Cell aggregates in each group were initially perfused in isosmotic control solution for 20 min, followed by another 20 min perfusion in solutions with different osmolarity, as indicated in the table. All preparations were finally reperfused in isosmotic solution for 20 min to allow for recovery. Total Na^+ and K^+ contents were measured after 20 min recovery in isosmotic solution. All solutions were Hepes–Tris-buffered solutions. Hyposmotic solutions were made by reducing NaCl concentration in isosmotic control solution (100% $[NaCl]_o$ contained 142·6 mM NaCl). n, the number of experiments; * statistically different from control, $P < 0.05$ using Student's unpaired t test.

electrical and contractile events demonstrates that such changes are not due to changes in the seal resistance of the microelectrode. Like the RVD, the rate and degree of depolarization appeared to be a function of the degree of hyposmotic challenge. Superfusion of the preparations with hyposmotic solutions containing

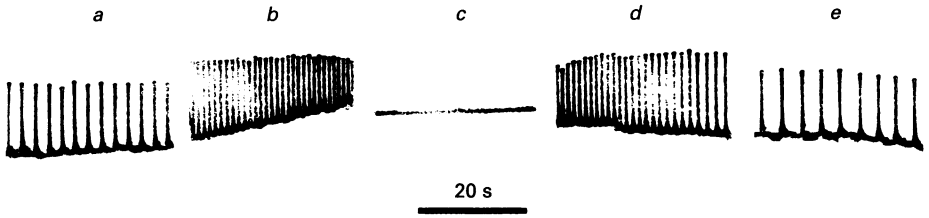

Fig. 2. The effect of cell swelling on the contractile activity of cultured chick heart cells. An edge detecting device was used to record the movement of a latex bead on the surface of contracting polystrand preparations (Methods). Polystrands were first perfused in isosmotic control solution for at least 10 min, then switched to hyposmotic solution (50% NaCl). After becoming quiescent in hyposmotic solution for ~2 min, the preparations were reperfused with isosmotic solution for the rest of the experiment. Results were obtained from a continuous recording of the same polystrand preparation. *a*, contractile activity under control condition; *b*, ~2 min in hyposmotic solution; *c*, ~3 min in hyposmotic solution; *d*, ~2 min after reperfusion in isosmotic solution; *e*, ~3 min in isosmotic solution. Upward deflection represents cell contraction and slow drift of the baseline reflects shift in position due to changes in the volume of the preparation.

$\leqslant$ 50% NaCl, caused depolarization to ~ −45 mV, whereas the depolarization in 70% NaCl had a slower rate of onset and did not always exceed threshold. Figure 3*B* plots the effect of hyposmotic solution on the diastolic potential of nineteen similar polystrand preparations. E_m was recorded continuously as the superfusate was switched from control to hyposmotic solution and back to control, and the subsequent depolarization was measured as the change from the diastolic potential under control conditions. Sodium chloride (70%) caused a significant depolarization of $12{\cdot}7 \pm 9{\cdot}2$ mV ($n = 7$); 50% NaCl depolarized the membrane by $35{\cdot}6 \pm 3{\cdot}8$ mV ($n = 5$) but 25% NaCl caused no further depolarization ($\Delta E_m = 34{\cdot}1 \pm 3{\cdot}8$ mV, $n = 7$).

Whole-cell current activated by hyposmotic cell swelling

The observed changes in spontaneous electrical and contractile activities during hyposmotic swelling are consistent with the activation of a depolarizing Cl^- conductance. However, changes in other time- and voltage-dependent currents can also alter the rates of spontaneous electrical activity in cardiac cells (e.g. K^+ or Ca^{2+} currents, see recent review by Campbell, Rasmusson & Strauss, 1992). Swelling-induced changes in membrane conductance were measured using whole-cell patch clamp experiments. Cells started to swell within 1–2 min after exposure to a hyposmotic solution. Cell volume increased continuously over a period of ~10 min to a relative volume (V/V_0) of $1{\cdot}31 \pm 0{\cdot}03$ ($3{\cdot}2 \times 10^3 \pm 0{\cdot}6 \times 10^3$ μm^3 *vs.* $4{\cdot}2 \times 10^3 \pm 0{\cdot}9 \times 10^3$ μm^3, $n = 30$; $P < 0{\cdot}001$). No apparent volume regulation was observed throughout the volume perturbation. Figure 4 illustrates the effect of

decreasing extracellular osmolarity on the whole-cell current. Changes in whole-cell current were monitored by applying 100 ms test pulses every 12 s to +60 mV from a holding potential of −40 mV. The holding current and the test current at +60 mV were plotted as a function of time. The inward holding current under control

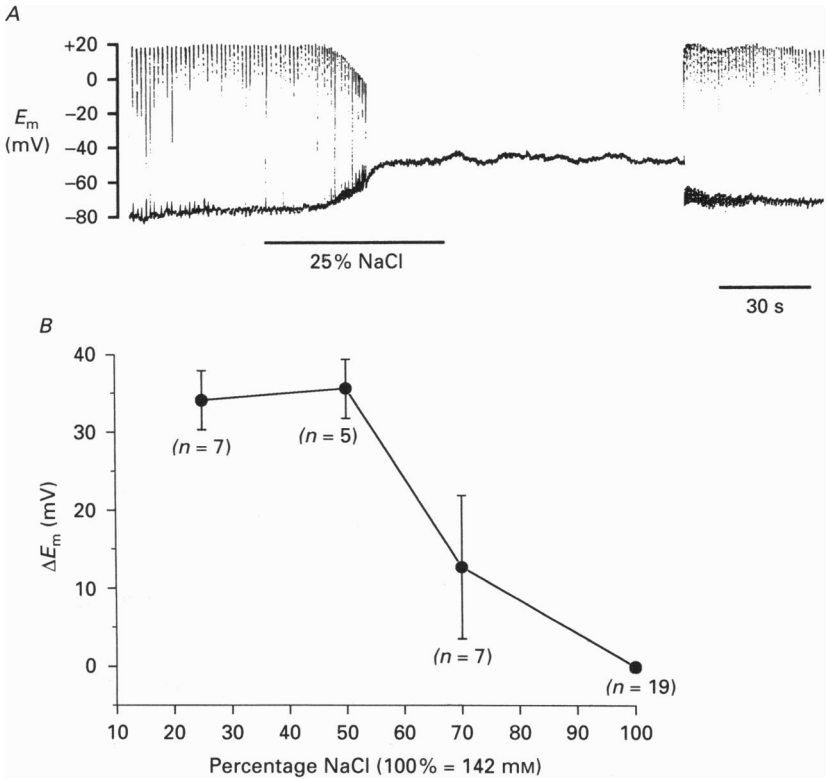

Fig. 3. The effect of cell swelling on the diastolic membrane potential of cultured chick heart cells in polystrand preparations. *A*, transmembrane potential recorded continuously from a spontaneously active preparation, using a conventional 3 M KCl microelectrode, during superfusion with hyposmotic solution (25% NaCl). *B*, depolarization of the cell membrane in hyposmotic solutions (low NaCl). Steady-state changes in E_m were measured from the diastolic membrane potential in isosmotic solution (100% NaCl). Data points represent the mean ± S.D. of the number (n) indicated.

conditions reflects an inward background current typical in spontaneously active cells. Hyposmotic swelling induced an outward current at +60 mV and an inward shift in holding current at −40 mV. Total membrane current increased continuously for the duration of the cell swelling. After ~ 10 min of perfusion in hyposmotic solution, the outward current increased by 11·5 ± 2·5 times the control current measured under isosmotic conditions (n = 12). Upon return to isosmotic solution the membrane current slowly recovered to initial control values.

Figure 5*A* and *B* demonstrates typical whole-cell current records under isosmotic and hyposmotic conditions, respectively. Swelling-induced changes in whole-cell

currents were obtained by subtracting the current records under isosmotic conditions from those obtained during cell swelling (Fig. 5*C*). The swelling-activated current showed no time dependence during the 100 ms voltage pulses. The current–voltage relationship of this swelling-induced current, as plotted in Fig. 5*D*, displayed

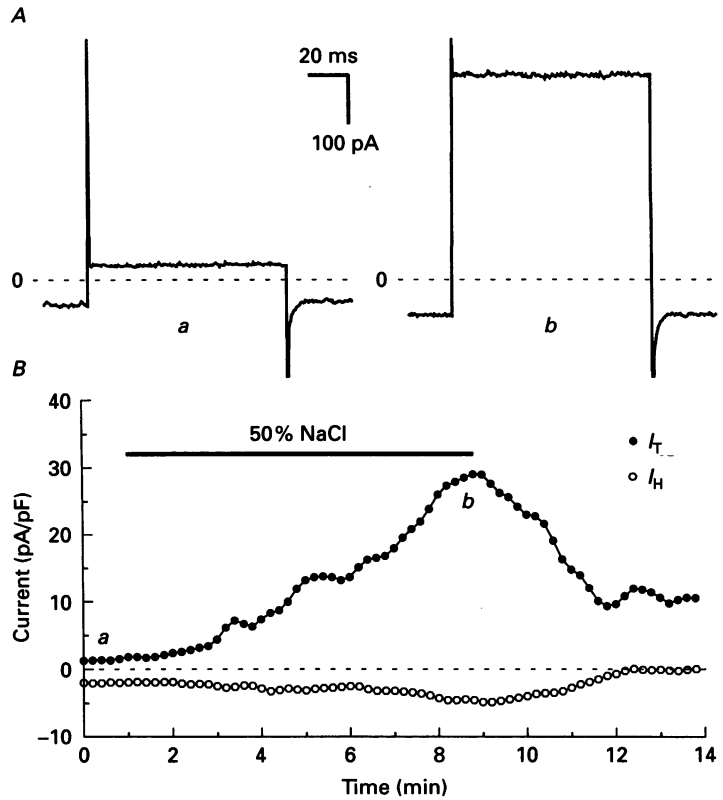

Fig. 4. Hyposmotically (50% NaCl) induced changes in membrane conductance at depolarized membrane potentials. Whole-cell current was elicited by applying 100 ms voltage clamp steps to +60 mV from a holding potential of −40 mV, at 12 s intervals. *A*, whole-cell currents obtained under isosmotic condition (*a*) and after hyposmotically induced cell swelling (*b*). *B*, time course of hyposmotically induced changes in whole-cell current. Currents at time points *a* and *b* are shown in *A*. I_T, current at test voltage of +60 mV; I_H, holding current at −40 mV.

outward rectification, similar to the Cl^- currents demonstrated in epithelial cells (Gogelein, 1988; Schoppa, Shorofsky, Jow & Nelson, 1989) and cardiac cells (Bahinski *et al.* 1989; Harvey *et al.* 1990; Matsuoka, Ehara & Noma, 1990; Sorota, 1992; Tseng, 1992), and those of swelling-activated Cl^- channels in other cell types (Cahalan & Lewis, 1988; Hudson & Schultz, 1988; Worrell, Butt, Cliff & Frizzell, 1989). In addition, the apparent reversal potential for this difference current was approximately $-23{\cdot}1 \pm 6{\cdot}7$ mV ($n = 5$), consistent with a Cl^--mediated conductance that is activated by cell swelling.

Since the reduction of osmolarity was associated with a reduction in the $[Na^+]_o$ of the bath solution, we tested the Na^+ dependence of the swelling-activated whole-cell conductance. Replacing Na_o^+ with NMDG either before or after hyposmotic challenge had no apparent effect on the swelling-activated current (J. Zhang,

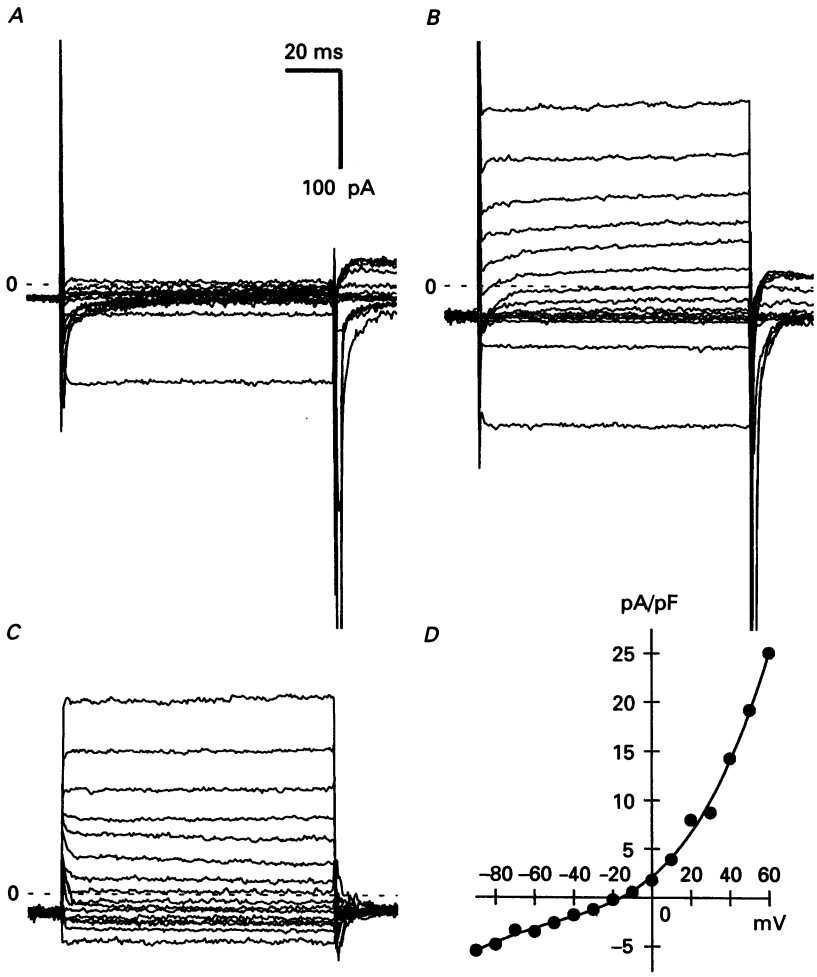

Fig. 5. Whole-cell current activated by hyposmotic (50% NaCl) swelling. Whole-cell current was elicited by applying 100 ms voltage steps to membrane potentials between −90 and +60 mV in 10 mV steps from a holding potential of −40 mV. *A*, whole-cell currents under isosmotic conditions. *B*, whole-cell currents during hyposmotic swelling. *C*, swelling-activated currents obtained by subtracting current records in isosmotic solution (*A*) from those recorded in hyposmotic solution (*B*). *D*, current–voltage relationship of the swelling-activated whole-cell current measured from the difference currents (*C*) at the steady state.

unpublished data). These results demonstrate that the swelling-activated current is insensitive to changes in $[Na^+]_o$.

Isolation of a swelling-activated Cl^- current

The swelling-activated current was further studied by blocking the K^+ conductances using Ba^{2+} or Cs^{2+} as a substitute for K^+ (see Methods). Voltage ramp

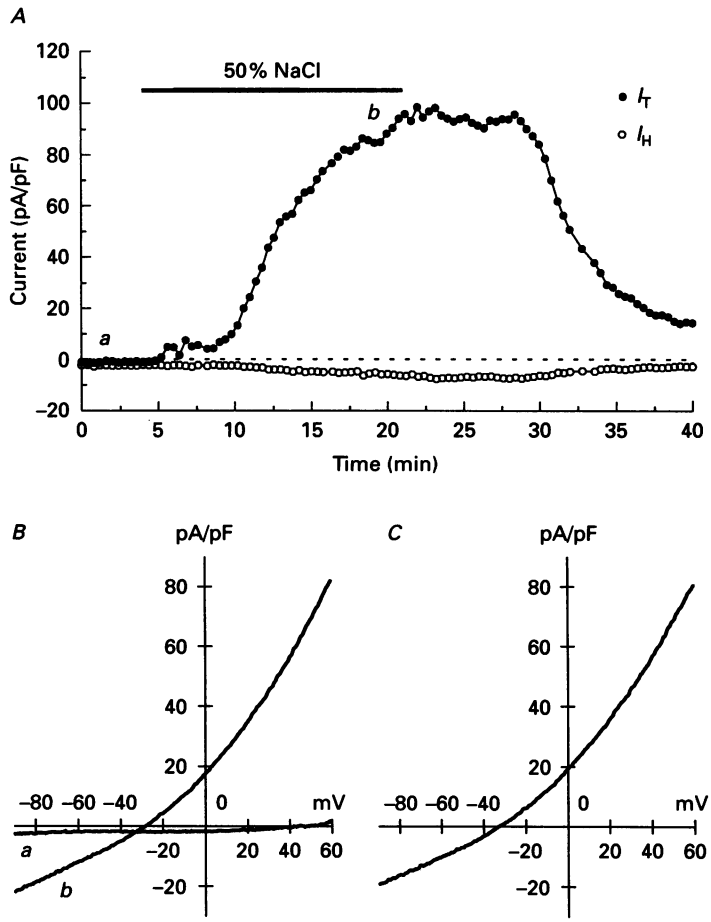

Fig. 6. Hyposmotically (50% NaCl) induced changes in membrane current by whole-cell patch clamp. Voltage ramps (−90 to +60 mV, $dV/dt = \pm 0{\cdot}5$ V/s) were applied at 12 s intervals from a holding potential of −40 mV. K^+ current was blocked by 1 mM Ba^{2+}. *A*, time course of whole-cell current. I_T, current at ramp voltage of +60 mV. I_H, holding current at −40 mV. *B*, current–voltage relationship obtained by ramp pulses at different time points as indicated (*a* and *b*). *C*, current–voltage relationship of hyposmotically activated current obtained by subtracting ramp *I–V* relation in isosmotic solution (*a*) from ramp *I–V* relation in hyposmotic solution (*b*).

protocols were used to measure the *I–V* characteristics of this current during experimental interventions because the currents did not show any time-dependent kinetics. Figure 6*A* shows the time course of changes in whole-cell current at the

ramp voltage of +60 mV (I_T) and the holding current (I_H) at −40 mV. Under these conditions, hyposmotically induced cell swelling caused an inward shift of the holding current accompanied by a substantial increase in the outward current measured at +60 mV, which is consistent with the difference currents without

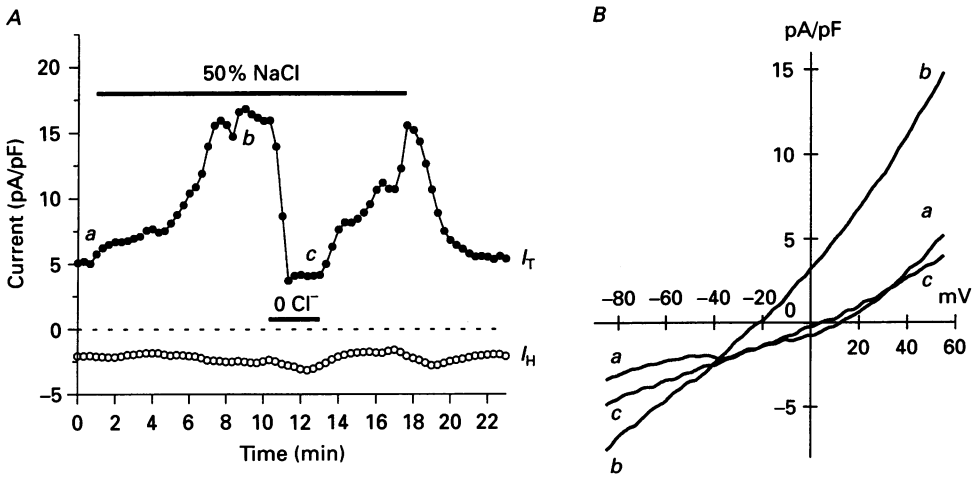

Fig. 7. Hyposmotically (50% NaCl) activated whole-cell current in the presence and absence of external Cl^-. Substitution with an equimolar concentration of methanesulphonate was used to obtain Cl^--free solutions. Voltage ramps (−90 to +60 mV, $dV/dt = \pm 0{\cdot}5$ V/s) were applied at 20 s intervals from a holding potential of −40 mV. K^+ current was blocked by 1 mM Ba^{2+}. *A*, time course of whole-cell current. I_T, current at ramp voltage of +60 mV. I_H, holding current (−40 mV). *B*, current–voltage relationship obtained by ramp pulses at different time points as indicated in *A* (*a*, *b* and *c*).

blocking K^+ conductance (Fig. 5). The current–voltage relationships obtained from voltage ramp pulses under isosmotic and hyposmotic conditions are illustrated in Fig. 6*B*. Control slope conductance in isosmotic solution was calculated to be $43{\cdot}5 \pm 7{\cdot}4$ pS/pF ($n = 17$) for the voltage range of −90 to −70 mV and was $222{\cdot}0 \pm 33{\cdot}8$ pS/pF ($n = 17$) between +40 and +60 mV. During cell swelling, however, the slope conductance of the same preparations increased to $261{\cdot}2 \pm 28{\cdot}9$ pS/pF and $1264{\cdot}9 \pm 121{\cdot}9$ pS/pF, respectively. The current–voltage relationship of the swelling-activated current was determined by subtraction of ramp *I–V* values between isosmotic and hyposmotic conditions (Fig. 6*C*). This difference *I–V* exhibited the basic characteristics similar to those obtained in the absence of K^+ channel blockers (Fig. 5), thereby indicating that neither the use of K^+ channel blockers nor the ramp clamp (as opposed to step clamp employed in Fig. 5) affected the hyposmotically activated current. The reversal potential of the difference current was $-24{\cdot}8 \pm 6{\cdot}4$ mV ($n = 6$), which is very close to both the equilibrium potential of Cl^- (E_{Cl}, −23·6 mV) and the reversal potential measured in the absence of K^+ channel blockers (−23·1 mV, see above). The hyposmotically activated current was reversed by reperfusion in isosmotic solution (Fig. 6*A*).

To demonstrate the Cl^- dependence of the swelling-activated current, Cl_o^- was replaced by methanesulphonate during hyposmotic cell swelling. This manipu-

lation resulted in a nearly complete abolition of the swelling-activated current at the test potential of +60 mV and a reduction of the slope conductance at +60 mV from 236·7 to 82·4 pS/pF, consistent with a high selectivity of this swelling-activated conductance to chloride (Fig. 7). The swelling-activated current was restored upon return to the Cl^--containing hyposmotic solution, and this current fully returned to control level during the recovery in isosmotic solution.

TABLE 3. Reversal potential of swelling-activated current in chick heart cells

$[Cl^-]_o$ (mM)	$[Cl^-]_i$ (mM)	E_{Cl} (mV)	E_{rev} (mV)	n
71·7	16·1	−40	−38·6 ±0·9	6
84·7	35·0	−23·6	−24·8 ±6·4	6
35·0	35·0	0	−5·3 ±3·1	4
82·7	150·0	15·9	8·3 ±0·6	5
33·6	150·0	40	26·7 ±10·1	5

E_{rev}, experimentally measured reversal potential; E_{Cl}, equilibrium potential of Cl^-. Values are presented as mean ± S.D. n, the number of experiments. $[Cl^-]_o$ was altered by replacing NaCl with an equiosmolar concentration of sucrose. $[Cl^-]_i$ was varied by substitution of chloride for aspartate to increase $[Cl^-]_i$, or vice versa to reduce $[Cl^-]_i$.

Based on changes in reversal potential by Cl^- substitution with methanesulphonate and aspartate, the ability of the swelling-activated anion conductance to carry larger organic ions was examined by applying the Goldman–Hodgkin–Katz voltage equation. Permeability ratios of the swelling-activated conductance to Cl^-, methanesulphonate (MSA) and aspartate (Asp) were calculated as 1:0·36 for P_{Cl}/P_{MSA} and 1:0·02 for P_{Cl}/P_{Asp} with the sequence of chloride > methanesulphonate ≫ aspartate.

The ion selectivity of the swelling-activated current for Cl^- over MSA^-, as estimated from changes in reversal potential, was also investigated by varying the internal and external Cl^- concentrations. Table 3 summarizes the measured reversal potentials of current–voltage relationships at various Cl^- gradients (sucrose substitution for external Cl^-). The relationship between the experimentally measured reversal potential (E_{rev}) of the swelling-activated current and the predicted Cl^- equilibrium potential (E_{Cl}) in Table 3 was evaluated by linear regression. The measured reversal potential closely followed the unity relationship expected for a pure Cl^- current, with a linear regression slope of 0·82, in contrast to a slope of 0·45 when $[Cl^-]_o$ was varied by substitution with methanesulphonate. Reversal potentials of the swelling-activated current appeared to be a logarithmic function of $[Cl^-]_i/[Cl^-]_o$. Linear regression analysis of reversal potential as a function of log $([Cl^-]_i/[Cl^-]_o)$ produced a slope of 51 mV per 10-fold change in the concentration ratio (51 mV/decade) when $[Cl^-]_o$ was varied by substitution with sucrose, whereas the slope decreased to 28 mV/decade when methanesulphonate was used as a substitute for Cl_o^-, consistent with a membrane conductance that is highly selective for Cl^-. The results suggest that Cl^- is the major charge carrier of the swelling-activated current and that the deviation of E_{rev} from E_{Cl} might be explained by the ability of the channel to pass other anions such as methanesulphonate and aspartate.

Further confirmation that the swelling-activated current was carried by Cl^- was obtained by observing the effects of diphenylamine-2-carboxylate (DPC), an

inhibitor of transmembrane Cl^- conductance (Di Stefano, Wittner, Schlatter, Lang, Englert & Greger, 1985; Greger, 1990). DPC (200 μM) substantially decreased the swelling-activated current without shifting the reversal potential (Fig. 8). The reduction of the holding current at -40 mV was $50 \pm 14\%$ ($n = 4$), and was partly

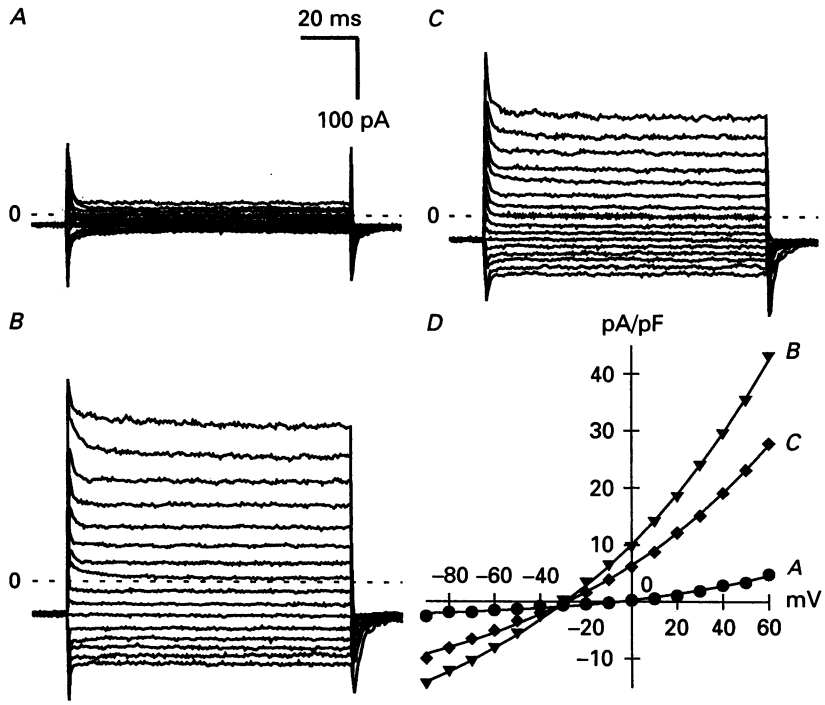

Fig. 8. Effect of the Cl^- channel blocker diphenylamine-2-carboxylate (DPC) on the hypo-osmotic (50% NaCl) swelling-activated current. Whole-cell current was elicited by applying 100 ms voltage steps to membrane potentials between -90 and $+60$ mV in 10 mV steps from a holding potential of -40 mV. K^+ current was blocked by 1 mM Ba^{2+}. *A*, whole-cell current under isosmotic condition. *B*, hyposmotic swelling-activated whole-cell current. *C*, whole-cell current of hyposmotically swollen cells after exposure to 200 μM DPC. *D*, current–voltage relationship of whole-cell currents shown in *A*, *B* and *C*.

reversible after a 5 min period of wash-out. A similar degree of inhibition was observed at all voltages (-90 to $+60$ mV). This observation is comparable to that reported with a similar concentration of DPC in Cl^--transporting epithelial cells (Di Stefano *et al.* 1985), and further supports the hypothesis that the hyposmotically activated current is a Cl^- current.

DISCUSSION

Hyposmotic cell swelling activates a sustained current that is reversible upon return to isosmotic solution. The observed increase in whole-cell conductance is carried by Cl^- under physiological conditions. The current is partly blocked in a

reversible fashion by DPC, a known inhibitor of Cl^- channels. This swelling-activated conductance may provide a Cl^- efflux pathway and contribute to the observed changes in spontaneous electrical activity associated with cell swelling.

Hyposmotically induced cell swelling

In whole-cell patch clamp experiments, cells continued to swell throughout exposure to hyposmotic solution, without undergoing any apparent volume regulation, i.e. cell volume never reached steady state during hyposmotic challenge. Several factors may contribute to the apparent lack of RVD in the whole-cell configuration. Firstly, the intracellular osmotic and ionic environments are buffered by the pipette filling solution, and the volume of the intracellular compartment is effectively extended towards infinity. When the extracellular solution is rendered hyposmotic water enters the cell by osmosis, but dilution of the intracellular solution is compensated by the ionic composition of the pipette solution. Hence the gradient for water influx is maintained, and the cell continues to swell beyond the expected volume change. Secondly, the absence of amino acids in the pipette filling solution is likely to prevent volume regulation by organic osmolyte loss from the cell. Similarly, dialysis of the cytoplasm in the whole-cell configuration can dilute intracellular signalling pathways so that volume regulatory mechanisms are compromised. This hypothesis is supported by the observation that cells demonstrated volume regulation when experiments were repeated using the nystatin-perforated patch clamp technique, in which the cells remain metabolically intact with only limited dialysis of monovalent ions (Hall, Zhang & Lieberman, 1993).

The fact that cells swell at all under whole-cell patch clamp is puzzling. If one considers that, in this configuration, the cell no longer represents a closed space and the cell membrane simply separates the large pools of intracellular and extracellular solutions, then movement of water between these pools would not be expected to affect cell volume. It is tempting to invoke more than simple water movement, e.g. cytoskeletal rearrangement and macromolecular crowding (Sarkadi & Parker, 1991) to explain swelling under these conditions.

Cl^- current and amino acid efflux during cell swelling

In cultured chick heart cells, the maximum diastolic potential is ~ -80 mV, whereas the Cl^- equilibrium potential is ~ -27 mV (Liu *et al.* 1987). Under physiological conditions, the background Cl^- conductance is negligible in cultured chick heart cells (Piwnica-Worms *et al.* 1983; present study) and unstimulated mammalian heart cells (Bahinski *et al.* 1989; Harvey *et al.* 1990). The outwardly directed Cl^- gradient is established primarily by the activity of the Na^+–K^+–2 Cl^--cotransporter (Liu *et al.* 1987). Consequently, Cl^- movement activated by cell swelling during the diastolic interval generates a net inward (depolarizing) current. This Cl^- current could contribute to the observed increase in spontaneous electrical and contractile activity and could be coupled with K^+ movement to produce a net efflux of KCl during the diastolic interval.

If electrodiffusive loss of KCl was the only mechanism of RVD, then based on the initial E_{Cl} of -27 mV which changed to ~ -40 mV during hyposmotic swelling (close to resting potential, see Fig. 3), the *maximum* RVD should be less than 16%

(i.e. loss of Cl^- activity is less than 22 mmol/l of original cell water), which is less than what is shown in Fig. 1. However, the parallel loss of amino acids, which has a concentrating effect on $[Cl^-]_i$, may enhance Cl^- loss during RVD. Therefore, we have attempted to address the relationship between measured changes in volume, amino acid content and Cl^- electrochemical equilibrium, based on the previously identified loss of amino acids during RVD (Rasmusson *et al.* 1993).

The loss of inorganic cations cannot be accounted for in terms of coupling to the loss of negatively charged aspartate and glutamate during RVD. For a reduction of osmolarity from 293 to 164 mosmol/l (50% NaCl) and 98 mosmol/l (25% NaCl), the amount of inorganic cation loss was 73 and 514 nmol/mg protein (Table 1), which compares favourably with the results under HCO_3^--buffered conditions (Rasmusson *et al.* 1993). When corrected for amino acid charge balance, the remaining loss of inorganic cations was 8 and 366 nmol/mg protein, equivalent to 1·1 and 49 mmol/l of original cell water (original cell water content = 7·45 μl/mg protein, Rasmusson *et al.* 1993), which is comparable to the measured intracellular Cl^- activity of approximately 35 mM.

The loss of Cl^- required to balance 49 mmol/l loss of inorganic cations seems large when compared with the 22 mM maximum loss of Cl^- activity calculated from changes in the reversal potential. However, this calculation was based on the assumption that RVD is totally mediated by Cl^-. The loss of organic and inorganic osmolytes would cause a reduction in cell volume to further concentrate the intracellular solutes. Given the final relative volume of 1·07 after 20 min exposure to a 25% NaCl hyposmotic solution (Rasmusson *et al.* 1993) and E_{Cl} of -40 mV, $[Cl^-]_i$ can be estimated from the Nernst equation (at 37 °C), assuming the intra- and extracellular activity coefficients are identical:

$$43 \text{ mM } (Cl_o^-) \exp[-40 \text{ mV}/(RT/F)] = 10 \text{ mM } (Cl_i^-),$$

where R is the gas constant, T is absolute temperature and F is the Faraday constant. Since cell volume is reduced from the initial swollen state, Cl_i^- content can be calculated as 10 mM × 7·45 μl/mg protein × 1·07 (V/V_0) = 80 nmol/mg protein. For comparison we can use identical assumptions in the Nernst equation to calculate $[Cl^-]_i$ in isosmotic solution:

$$150 \text{ mM } (Cl_o^-) \exp[-27 \text{ mV}/(RT/F)] = 55 \text{ mM } (Cl_i^-),$$

which is equivalent to a Cl_i^- content of 55 mM × 7·45 μl/mg protein × 1·00 (V/V_0) = 410 nmol/mg protein. The calculated decline in Cl^- content ($\sim$330 nmol/mg protein) compares remarkably well with the inorganic cation losses reported in Results. Therefore, it is likely that the concentrating effect of loss of amino acids during RVD enhances the volume regulatory KCl efflux by increasing the outward chemical potential for these ions.

The swelling-activated Cl^- conductance and other transport mechanisms

As mentioned earlier, basal Cl^- conductance in unstimulated cardiac cells is low (Piwnica-Worms *et al.* 1983; Bahinski *et al.* 1989; Harvey *et al.* 1990); but the basal permeability to Cl^- is relatively high (Piwnica-Worms *et al.* 1983; Liu *et al.* 1987). In Hepes–Tris buffer, the high permeability to Cl^- is primarily due to the

Na^+–K^+–2 Cl^- cotransporter, a relatively large capacity electroneutral transporter believed to operate near its thermodynamic equilibrium (Liu *et al.* 1987). In addition, the Cl^-–HCO_3^--coupled transport mechanisms involved in pH regulation of cardiac muscle are inhibited at the ambient levels of bicarbonate present in most non-bicarbonate-buffered solutions (Liu *et al.* 1990). Thus, for the purposes of our discussion, the inwardly directed Na^+–K^+–2 Cl^- cotransporter is the major background transporter establishing Cl^- gradients.

Other studies in cardiac cells have implicated coupled Cl^- transport in the volume regulatory process (Drewnowska & Baumgarten, 1991). We cannot rule out the possibility that changes in coupled transport mechanisms occur in parallel with the activation of a volume-sensitive Cl^- current. For example, RVD may be enhanced if the Na^+–K^+–2 Cl^- cotransporter is inactivated during the activation of Cl^- channels.

Reduction of the extracellular Na^+ concentration in hyposmotic solution may suppress the activity of Na^+-dependent transport mechanisms. As a consequence, the intracellular milieu would be altered, and it would be difficult to determine if the activation of Cl^- current is due solely to cell swelling. However, it seems unlikely that external Na^+ was involved in modulating the swelling-activated Cl^- current because no significant change in the steady-state whole-cell current was observed when the Na^+ gradient was altered in either isosmotic or hyposmotic solutions (J. Zhang, unpublished data). In addition, suppression of these Na^+-dependent transporters by reduction of the external Na^+ concentration during hyposmotic perfusion will favour the RVD by reducing the net influx of ionic osmolytes. Therefore activation of Cl^- conductance during hyposmotic swelling might be underestimated if these Na^+-dependent transporters were inhibited.

Comparison with other cardiac Cl^- channels

Chloride channel activity in cardiac muscle has been observed in response to two different stimuli, β-adrenergic stimulation and acute cell swelling. Whether these two chloride currents represent different modulation of the same channel is currently under debate. Sorota (1992) argued that isoprenaline sensitivity in canine atrial cells is only conferred after cell swelling has been induced through prolonged dialysis inherent in the whole-cell patch clamp mode or following hyposmotic exposure. Conversely, the isoprenaline-activated Cl^- current observed in guinea-pig atrial and ventricular cells (Bahinski *et al.* 1989; Harvey & Hume, 1989; Harvey *et al.* 1990; Matsuoka *et al.* 1990) and in rabbit ventricular cells (Harvey & Hume, 1989) is fully reversible, arguing against the hypothesis of prolonged dialysis. Furthermore, our preliminary data indicate that the basal conductance of chick heart cells was insensitive to isoprenaline or forskolin and the swelling-activated Cl^- conductance was suppressed by these agents (Zhang, Smith, Lobaugh, Hall & Lieberman, 1992*c*). This finding challenges the possibility that the volume-activated Cl^- channel is activated by β-adrenergic stimulation. Thus, in cultured chick heart cells the volume-activated chloride channel seems to be distinct from the isoprenaline-activated Cl^- channel observed in mammalian cardiac preparations.

Possible role of the swelling-activated Cl^- conductance in cardiac physiology

The characteristics of the swelling-activated Cl^- conductance in cultured chick heart cells described in this study support the hypothesis that the volume-activated Cl^--conductive pathway promotes KCl efflux during cell swelling. Although no evidence for a volume-sensitive K^+ conductance has been reported in cardiac cells, the K^+ conductance (inward and delayed rectifier) normally present in cultured chick heart cells may provide a parallel pathway for K^+ efflux during RVD.

The presence of RVD in cultured chick heart cells is at variance with data obtained from volume measurements of freshly isolated single rabbit ventricular cells exposed to hyposmotic solutions, in which no apparent time-dependent volume regulatory decrease was reported (Drewnowska & Baumgarten, 1991). In adult cardiac myocytes, regulatory mechanisms may be more sensitive to changes in cell volume; thus, these cells may undergo more immediate volume regulation, so that the initial swelling is not observed.

The physiological role of the swelling-activated Cl^- conductance remains controversial, because the osmolarity of extracellular fluid is normally unchanging *in vivo*. However, perturbation of cell volume could arise from an imposed change in intracellular solute content under pathophysiological conditions. Failure of cell volume control associated with myocardial ischaemia (Jennings *et al.* 1986) and cardioplegic manoeuvres (Drewnowska, Clemo & Baumgarten, 1991) results in an increase in myocyte volume and, if prolonged, cardiac cell injury. Activation of a Cl^- conductance in response to cell swelling and the subsequent depolarization of membrane potential could prevent or delay irreversible cell injury by offsetting the increase in intracellular solute content. Alternatively, activation of the Cl^- conductance may contribute to rhythmic modulation of cardiac electrical activity under physiological conditions, because the swelling-activated Cl^- conductance can also be induced by membrane stretch (Hagiwara *et al.* 1992).

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