Hypotonicity Activates a Native Chloride Current in *Xenopus* Oocytes

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ABSTRACT *Xenopus* oocytes are frequently utilized for in vivo expression of cellular proteins, especially ion channel proteins. A thorough understanding of the endogenous conductances and their regulation is paramount for proper characterization of expressed channel proteins. Here we detail a novel chloride current ($I_{Cl.swell}$) responsive to hypotonicity in *Xenopus* oocytes using the two-electrode voltage clamp technique. Reducing the extracellular osmolarity by 50% elicited a calcium-independent chloride current having an anion conductivity sequence identical with swelling-induced chloride currents observed in epithelial cells. The hypotonicity-activated current was blocked by chloride channel blockers, trivalent lanthanides, and nucleotides. G-protein, cAMP-PKA, and arachidonic acid signaling cascades were not involved in $I_{Cl.swell}$ activation. $I_{Cl.swell}$ is distinct from both stretch-activated nonselective cation channels and the calcium-activated chloride current in oocytes and may play a critical role in volume regulation in *Xenopus* oocytes.

INTRODUCTION

All cells possess mechanisms to regulate their volume precisely during mitosis and osmotic challenge (see recent reviews by Hoffmann and Simonsen, 1989; Sarkadi and Parker, 1991; Chamberlin and Strange, 1989; Lewis and Donaldson, 1990). Most cells swell after being placed in a hypotonic environment but over the course of several minutes shrink back toward their initial volume via a complex process known as regulatory volume decrease (RVD). The cell is able to undergo RVD by losing intracellular potassium and chloride ions accompanied by an obligatory loss of water. Several transport mechanisms including independent potassium and chloride conductive systems have been implicated in RVD (Hoffmann and Simonsen, 1989).

Amphibian oocytes regulate their volume during both hypotonic (Berntsson, Haglund, and Lovtrup, 1965) and hypertonic exposure (Dick and Dick, 1970). Berntsson et al. (1965) demonstrated a 20% increase in the volume of ovarian eggs from *Rana temporaria* within 1 h after exposure to a hypotonic (50% Ringer's) solution; RVD was complete within 24 h. They suggested that RVD was achieved, independent of extracellular calcium, through the generation of mechanical tension in the vitelline membrane (ultrafiltration). Indirect measurements indicated that the

intracellular pressure increased 10-fold in these oocytes, from 0.02 mm Hg in 220 mOsm Ringer's solution to 0.2 mm Hg in 50% (110 mOsm) Ringer's solution (Mild, Lovtrup, and Bergfors, 1974). Direct measurements of intracellular pressure in *Xenopus* oocytes (Kelly and Macklem, 1991) showed a 15-fold increase in intracellular pressure, from 0.2 mm Hg to 3.0 mm Hg, after a solution change from 200 to 60 mOsm, accompanied by a 24% increase in volume. Kelly and Macklem (1991) concluded, however, that the elastic properties of the vitelline membrane alone were insufficient to drive osmoregulation. In oocytes of *Rana temporaria*, restoration of normal volume in the continued presence of hypotonicity was achieved by the extrusion of potassium chloride (Sigler and Janacek, 1971).

The *Xenopus* oocyte exhibits several endogenous currents (see Dascal, 1987). Two conductances which could subserve an osmoregulatory function include stretch-activated/mechanosensitive channels (Methfessel, Witzemann, Takahashi, Mishina, Numa, and Sakmann, 1986; Yang and Sachs, 1989) and calcium-activated chloride currents ($I_{Cl,Ca}$) (Barish, 1983). Stretch-activated (SA) channels were described as 28-pS nonselective, cation channels ($E_{rev} = -10$ mV) in normal Ringer's solution (Methfessel et al., 1986). Yang and Sachs (1989) demonstrated reversible inhibition of SA-channels by the trivalent cations, gadolinium and lanthanum. Activation of SA-channels required at least 20–30 mm Hg of pressure applied to the membrane patch (Yang and Sachs, 1989). Recently, hypotonicity was reported to cause a twofold increase in the calcium-activated, outwardly rectifying chloride current (Chen, Chen, Kempson, and Yu, 1993). We provide evidence that hypotonicity activates a novel current in *Xenopus* oocytes.

Xenopus oocytes are commonly used for heterologous expression of proteins, particularly those involved in ion transport (Gurdon, Lane, Woodland, and Marbaix, 1971; Dascal, 1987; Lester, 1988; Sigel, 1990). Several proteins involved in chloride conduction have been expressed in oocytes including the cystic fibrosis transmembrane regulator CFTR (Bear, Duguay, Naismith, Kartner, Hanrahan, and Riordan, 1991), voltage-dependent chloride channels ClC-0, ClC-1, ClC-2 (Jentsch, Steinmeyer, and Schwarz, 1990; Steinmeyer, Ortland, and Jentsch, 1991; Thiemann, Grunder, Pusch, and Jentsch, 1992), ClCK1 (Uchida, Sasaki, Furukawa, Hiraoka, Imai, Hirata, and Marumo, 1993), I_{Cln} (Paulmichl, Li, Wickman, Ackerman, Peralta, and Clapham, 1992), and phospholemman (Moorman, Palmer, John, Durieux, and Jones, 1992). Effects of hypotonicity on CFTR (Hasegawa, Skach, Baker, Calayag, Lingappa, and Verkman, 1992) and ClC-2 (Grunder, Thiemann, Pusch, and Jentsch, 1992) have also been examined in oocytes. Our discovery of an endogenous chloride current responsive to hypotonicity requires that hypotonicity-related functions be assigned carefully to heterologously-expressed proteins.

MATERIALS AND METHODS

Xenopus laevis Oocytes

Adult female *Xenopus laevis* toads were obtained from Xenopus I (Ann Arbor, MI) and Nasco (Fort Atkinson, WI). Ovarian lobes were removed under sterile conditions from anesthetized toads. Harvested oocytes were kept at 19°C in ND96 (220 mOsm) solution containing in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES; pH was adjusted to 7.5 with NaOH. Osmolality

of all solutions was determined using a 5500 Vapor Pressure Osmometer and all abbreviations as mOsm refer to osmolality (Wescor Inc., Logan, UT). The incubation media was supplemented with 50 mg/liter gentamicin (Gibco Laboratories, Grand Island, NY) and 250 µg/liter amphotericin B (Gibco Laboratories) and was changed daily. Stage V–VI oocytes were manually defolliculated ~2 h after surgery. Oocytes defolliculated by collagenase treatment failed to activate $I_{Cl.swell}$ in response to an osmotic challenge (data not shown). Except where noted, defolliculated oocytes were maintained in the antibiotic-supplemented media for at least 12 h before electrophysiological recordings were made.

Electrophysiology

The two-electrode voltage clamp technique was used to measure the hypotonicity-activated chloride current in *Xenopus* oocytes using a Turbo TEC 01C from NPI Instruments (Tamm, Germany). Current and potential measuring electrodes were pulled (Flaming/Brown micropipette puller-Sutter Instrument Co., Novato, CA) having resistances of 1–3 MΩ and 2–5 MΩ, respectively when filled with 2M KCl. Current was measured as current flowing to ground through a low resistance electrode containing 2% agarose in 2M KCl. A second reference electrode was used to avoid polarization errors. Current measurements were filtered at 1 kHz. Data acquisition and analysis were done on a 80386-based microcomputer using pClamp software and TL-1 A/D converter (Axon Instruments, Burlingame, CA). All experiments were performed at room temperature. Each experiment began with measuring the basal current for the oocyte bathed in ND96 (220 mOsm) solution. Oocytes were kept in current clamp for at least 2 min after impalement with the measuring electrodes. Only oocytes exhibiting a resting membrane potential more hyperpolarized than −30 mV were used. Oocytes were clamped at a holding potential (V_h) of −70 mV, and 800 ms voltage steps from −90 to +70 mV in 20-mV increments were applied. This pulsing protocol was utilized for all experiments shown unless noted otherwise. Summarized data are presented as mean ± SEM unless otherwise indicated.

Extracellular hypotonicity was established by perfusing a hypotonic (110 mOsm) solution (ND48) containing in mM: 48 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES (pH 7.5) into the 500 µL chamber by gravity flow. Complete solution change (~10 bath vol) was achieved in under 2 min. Junction potentials resulting from solution changes were minimal, 1.5 ± 0.9 (SD) $n = 12$, and were not corrected. The voltage-clamp protocol was applied to the oocyte starting 1 min after hypotonic exposure. Characterization of this current was made without leak subtraction. Early experiments (not shown) using a p/4 leak-subtraction protocol from −70 mV resulted in a current profile that was markedly rectifying. However, the effects of chloride channel blockers demonstrated that leak subtraction inappropriately removed a linear component of inward chloride current. Thus, leak subtraction was not used. Tail current measurements were made using a 250-ms preconditioning pulse to +60 mV from $V_h = -70$ mV followed by 500-ms voltage steps from −120 to +40 mV in 20-mV increments.

Extracellular hypotonicity in previous characterizations of epithelial swelling-induced chloride currents was achieved by diluting an isotonic solution with water (Yantorno, Carre, Coca-Prados, Krupin, and Civan, 1992; Banderali and Roy, 1992), by removing mannitol or sucrose while maintaining equal ionic strength (Deiner, Nobles, and Rummel, 1992; Weiss and Lang, 1992; Kubo and Okada, 1992; Grunder et al., 1992), or by reducing sodium chloride, NMDG-glutamate, or NMDG choline (Worrell, Butt, Cliff, and Frizzell, 1989; McCann, Li, and Welsh, 1989; Doroshenko and Neher 1992; Chan, Goldstein, and Nelson, 1992; Valverde, Diaz, Sepulveda, Gill, Hyde, and Higgins, 1992). $I_{Cl.swell}$ was characterized using the third approach to allow direct comparison with T-84 colonic cells, airway epithelial cells, chromaffin cells, and P-glycoprotein expression. Experiments using sucrose to adjust the osmolality without changing ionic strength were also performed.

The extracellular concentration of chloride, potassium, and sodium were changed to

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examine the ion selectivity of $I_{Cl,swell}$. The hypotonicity-activated current was always measured in the standard hypotonic (ND48) solution both before and after a 2-min perfusion with the test hypotonic solution to ensure that the current was stable. Each test hypotonic solution varied in ionic composition but maintained the same reduced osmolality (110 mOsm). The test solutions included: high (50 mM) potassium, choline substitution for sodium, 50% sodium chloride with osmolality maintained by mannitol, low (7.6 mM) chloride, 48 mM NaX- (X- = thiocyanate, iodide, nitrate, bromide, methylsulphonate, bicarbonate, acetate, gluconate, and glutamate), and ND48 solution at varying pH.

Pharmacology of $I_{Cl,swell}$

After achieving maximal activation of $I_{Cl,swell}$, pharmacologic agents were added directly to the bath as 100-fold dilutions from freshly prepared stock solutions. The same voltage-clamp protocol was used after a 90-s drug exposure. At the end of each experiment, the drug was washed out with ≥ 10 bath vol (2 min). None of the agents tested affected the basal isotonic current. Therefore, the amplitude of the basal isotonic current at +70 mV was subtracted from the peak hypotonic current to normalize the dose response curves: $[I_{drug}-I_{isotonic}]/[I_{hypotonic}-I_{isotonic}]$. NPPB; 5-nitro-2-(3-phenylpropylamino)-benzoic acid (courtesy of R. Greger, University of Freiburg, Freiburg, Germany), DIDS; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (Sigma Chemical Co., St. Louis, MO), SITS; 4-acetamido-4'-isothiocyanastilbene-2,2'-disulfonic acid (Sigma Chemical Co.), and niflumic acid (Aldrich Chemical Co., Milwaukee, WI) were all dissolved in dimethylsulfoxide, DMSO (Sigma Chemical Co.). The final concentration of DMSO was $\leq 0.1\%$ which had no effect on the hypotonicity-activated current ($n = 7$). Arachidonic acid and leukotriene D_4 (Cayman Chemical Co., Ann Arbor, MI) were dissolved in ethanol. Lanthanum chloride (Fisher Scientific, Pittsburgh, PA), gadolinium chloride (Alfa Products, Danvers, MA), and cAMP (Sigma Chemical Co.) were dissolved in the standard hypotonic solution.

RESULTS

Hypotonicity Activates an Endogenous Current

The membrane potential of oocytes determined 3 min after impalement by the measuring electrodes was -44 ± 8 (SD) mV ($n = 128$). Fig. 1A demonstrates the ability of the oocyte to restore its membrane potential to nearly -70 mV when left in the isotonic solution for 15 min. Changing the extracellular chloride concentration from 103.6 mM to 55.6 mM (Fig. 1A; *shaded box*) had a negligible effect on the membrane potential, but further reduction to 7.6 mM chloride changed the membrane potential by 12.4 ± 2 mV (Fig. 1, A and C; $n = 5$) under isotonic conditions. A 10-fold increase in extracellular potassium shifted the membrane potential by 15 ± 1.2 mV (Fig. 1A, $n = 6$) suggesting that a mixture of potassium, chloride, and nonspecific leak pathways establish the oocyte's membrane potential in isotonic conditions.

In the isotonic (220 mOsm) bath, *Xenopus* oocytes have a small background current averaging -89 ± 5 nA at -90 mV and 420 ± 15 nA at $+70$ mV with a slope conductance of 1.6 μS at -30 mV ($n = 130$ oocytes, $N = 14$ toads, Fig. 2, A(1) and B). After determining the basal isotonic current, the standard hypotonic solution (110 mOsm) was perfused into the oocyte chamber. Fig. 2A(2) shows a representative response seen in 191/193 oocytes from 17 different toads exposed to the hypotonic solution. Only 5/193 (2.6%) oocytes displayed this current in isotonic conditions.
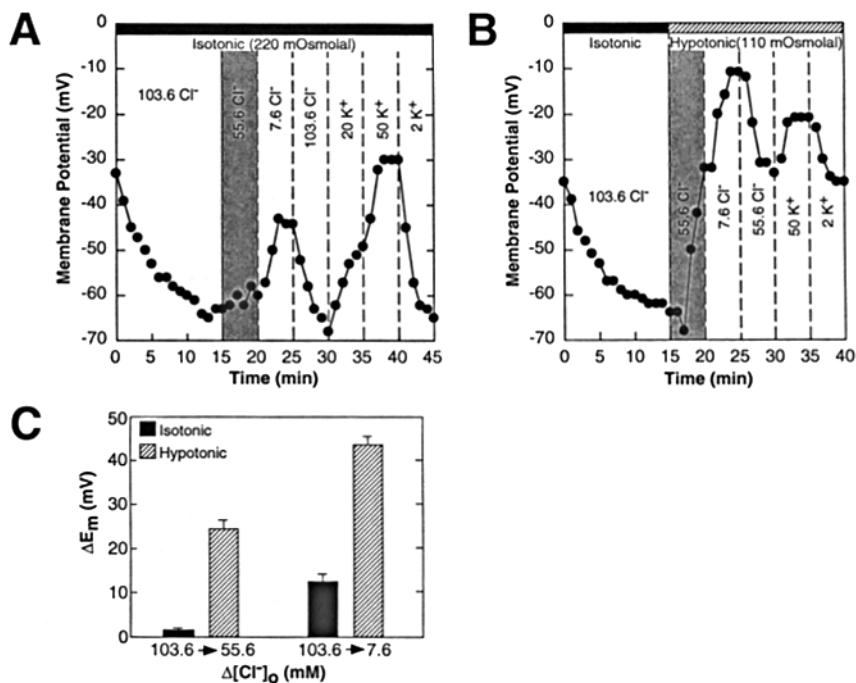
FIGURE 1. Hypotonic solution increases membrane chloride sensitivity in *Xenopus* oocytes.

(*A*) Oocyte membrane potential was monitored in current clamp over time during exposure to isotonic (220 mOsm) solutions containing different chloride and potassium concentrations. The oocyte's membrane potential recovered to nearly −70 mV during the 15 min after impalement of the measuring electrodes presumably due to "healing" of the membrane and restoration of the ion gradients. Changing extracellular chloride concentration caused only slight changes in the membrane potential with 55.6 mM chloride shifting the membrane potential from −63 mV to −60 mV. Increasing potassium from 2 mM to 20 mM decreased the membrane potential from −68 to −49 mV.

(*B*) Oocyte membrane potential as monitored in current clamp again recovered over time in the isotonic solution. Perfusion of the hypotonic (110 mOsm) solution resulted in a dramatic increase in membrane chloride sensitivity with 55.6 mM chloride shifting the membrane potential from −64 mV to −32 mV (compare with *A*). Decreasing the chloride concentration to 7.6 mM reduced the membrane potential to −11 mV. The hypotonically-activated conductance pathway mediating this increased chloride sensitivity also sensed changes in potassium but to a much lesser degree. A 25-fold increase in extracellular potassium changed the membrane potential by 12 mV.

(*C*) Summary of membrane chloride sensitivity determined during exposure to isotonic (*solid bars*) bath solutions and hypotonic (*hatched bars*) bath solutions. In isotonic media, changing the chloride concentration from 103.6 to 55.6 mM produced a 1.4 ± 0.6 mV ($n = 5$) change in membrane potential whereas the same change in extracellular chloride during hypotonic exposure resulted in a 24 ± 2 mV ($n = 5$) shift in membrane potential. Lowering the concentration to 7.6 mM changed the membrane potential by 43 ± 2 mV ($n = 7$) in hypotonic media compared to 12.4 ± 2 mV ($n = 5$) in isotonic media.

Exposure to the hypotonic solution induced a fast activating (<1 ms), slowly inactivating ($\tau_{off} = 484 \pm 11$ ms at $+70$ mV, $n = 86$), slightly rectifying current ($I_{Cl.swell}$) reversing between -25 and -35 mV. The current reached its peak after five minutes (4.4 ± 0.3 min; $n = 42$) in the hypotonic solution. The current-voltage
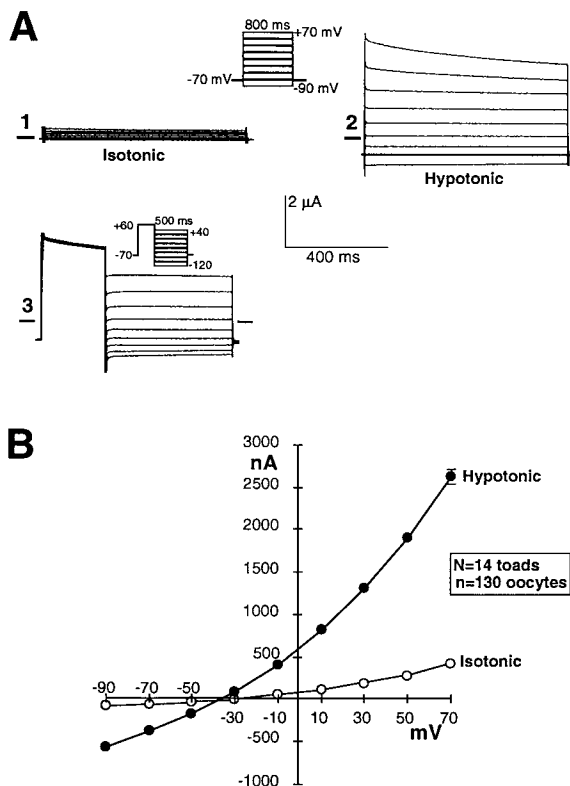

FIGURE 2. Hypotonic solution activates a novel current in *Xenopus* oocytes.

(*A*) Electrophysiological recordings using two-electrode voltage clamp on a *Xenopus laevis* oocyte. All measurements were made 12–48 h after manual defolliculation. After determining the background current (1) in isotonic (220 mOsm) conditions, oocytes were perfused with a hypotonic (110 mOsm) solution. 800-ms pulses from -90 to $+70$ mV in 20 mV steps from a holding potential of -70 mV elicited a sevenfold increase in current after 5 min in the hypotonic solution (2). The current was voltage-independent and displayed time dependent inactivation only at membrane potentials $> +30$ mV. The time dependence of inactivation at $+70$ mV was 484 ± 11 ms ($n = 85$). Tail currents (3) were determined for the hypotonicity-activated current after a 250-ms conditioning pulse to $+60$ mV from $V_h = -70$ mV. 500-ms test potentials from -120 to $+40$ mV demonstrate a reversal potential of approximately -25 mV. Horizontal lines indicate zero current.

(*B*) Current-voltage relationship of endogenous currents before (*open circles*) and during (*closed circles*) exposure to hypotonic solution. Currents were measured 30 ms into the test pulse and are shown as mean ± SEM for 130 oocytes from 14 different toads. Under isotonic conditions, current at -90 and $+70$ mV was -89 ± 5 nA and 420 ± 15 nA, respectively. Exposure to the hypotonic solution increased the current to -567 ± 21 nA and $2,628 \pm 90$ nA at -90 and $+70$ mV respectively, and increased the slope conductance at -30 mV from 1.6 to 16 μS. The swelling-induced current measured in this manner reversed between -30 and -40 mV.

relationship for this hypotonicity-activated current (*closed circles*) is shown in Fig. 2 *B*. Hypotonicity elicited a $>$ sixfold increase in current to -569 ± 21 nA and $2,629 \pm 89$ nA at -90 and $+70$ mV, respectively ($n = 130$ oocytes, $N = 14$ toads, ±SEM). The slope conductance at -30 mV increased by 10-fold to 16.8 μS despite a two-fold

reduction in extracellular chloride concentration. In addition to activating an endogenous conductance, perfusion of the hypotonic solution also shifted V_m from -44 ± 8 (SD) mV to -32 ± 5 (SD) mV ($n = 105$). Note that when the oocyte was first allowed to repolarize in the isotonic solution as in Fig. 1 *A*, exposure to the hypotonic solution which contained 55.6 mM chloride instead of 103.6 mM chloride caused a dramatic shift in the membrane potential from -64 mV to -32 mV within 5 min (Fig. 1 *B*, note *shaded box*). Again, a brief delay (2 min) occurred before the membrane potential depolarized in response to the hypotonic solution. This change in chloride concentration produced a 24 ± 2 mV change in membrane potential during exposure to the hypotonic solution in contrast to 1.4 ± 0.6 mV in isotonic media (Fig. 1 *C*, $n = 5$ each). This suggested that the chloride sensitivity of the oocyte membrane was increasing in response to hypotonicity.

Hypotonicity Does Not Activate Cation-selective Currents

The reversal potential (~ -30 mV) of the hypotonically-activated current suggested that hypotonicity activated either a chloride conductance or a nonselective cation conductance. Given the dramatic increase in membrane chloride sensitivity elicited after perfusion of a hypotonic solution (Fig. 1), it seemed probable that chloride channels were responsible for the hypotonicity-activated current (Fig. 2). The hypotonicity-activated current was minimally affected by changes in extracellular cations (Fig. 3). Replacing all extracellular sodium with choline had no effect on the osmosensitive current (Fig. 3 *A*[2] and *C* [$n = 5$]). Increasing the extracellular potassium concentration from 2 to 50 mM ($V_h = -70$ mV) increased the inward current (Fig. 3 *A*[3]) in a reversible manner (Fig. 3 *A*[4]) and shifted the reversal potential only slightly. The current-voltage relationship in Fig. 3 *B* summarizes the effects of extracellular potassium ($n = 11$). The peak inward current increased from -484 ± 32 nA in 2 mM K^+ (*open circles*) to -924 ± 66 nA (*closed circles*) in 50 mM K^+ whereas the outward current was unaffected. The 25-fold change in potassium shifted the reversal potential by 8 mV (Fig. 3 *B*). Moreover, perfusion of a hypotonic solution containing 24 mM NaCl (osmolality balanced with mannitol) resulted in a 9 ± 1.5 (SD) mV ($n = 4$, data not shown) shift in the depolarizing direction in both the membrane potential and reversal potential which fully reversed after returning again to the original 48 mM NaCl solution. If we assume that intracellular ion concentrations are unchanged during hypotonicity, calculations using the Goldman, Hodgkin, Katz equation demonstrate that P_{Cl}/P_{Na} is > 3. If intracellular concentrations of ions are diluted during swelling, then relative chloride selectivity is even greater. These experiments rule out swelling-activated cation currents that are selective between Na, K, and choline but are consistent with a chloride-permeant pathway.

Hypotonicity-activated Current Is a Chloride Current

Given the current's reversal potential, the sensitivity of V_m to chloride during hypotonicity, the minimal effect of extracellular cations, and an estimated $P_{Cl}/P_{Na} > 3$, it seemed likely that hypotonic solutions activated a chloride-permeable conduction pathway. This was established by examining the effects of extracellular chloride (Fig. 4) and chloride channel blockers (Fig. 5) on the hypotonicity-activated current. Lowering the extracellular chloride from 55.6 mM to 7.6 mM, by replacement of 48
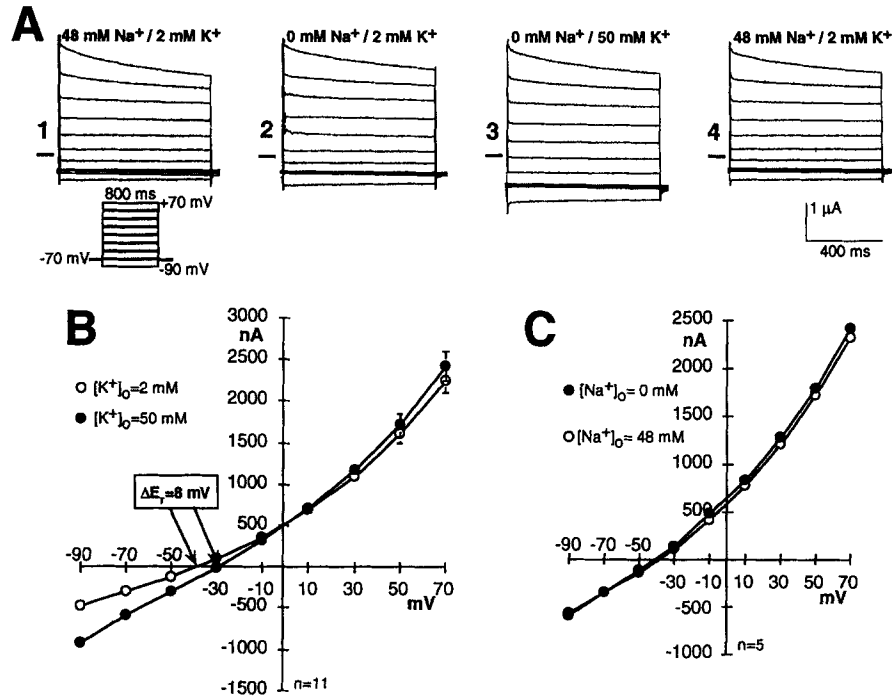
FIGURE 3. Hypotonicity-activated current is not comprised of stretch-activated cation channels.

(A) Electrophysiological recording after stimulation of current (1) in standard hypotonic solution (mM; 48 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, pulsing protocol shown, V$_h$ = -70 mV) Perfusion with a hypotonic solution containing choline in place of sodium (mM; 48 choline chloride, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 HEPES) produced no change in current magnitude or reversal potential (2). After the choline solution, a 25-fold increase in extracellular potassium (mM; 50 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 HEPES) caused a slight rightward shift in reversal potential and doubled the inward current (3). These effects reversed upon returning to the original (mM; 48 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 HEPES) hypotonic bathing solution (4). Horizontal lines indicate zero current.

(B) Current-voltage relationship showing the effects of changes in extracellular potassium concentration from 2 (open circles) to 50 mM (closed circles). After hypotonicity activated the endogenous current, peak inward current at -90 mV was -484 ± 32 nA in 2 mM KCl increasing to -924 ± 66 nA in 50 mM KCl. Outward current was unaffected. The reversal potential was shifted in the depolarizing direction by 8 ± 0.7 mV ($n = 11$).

(C) Average peak current-voltage relationship demonstrates the insensitivity of the reversal potential and current amplitude to changes in extracellular sodium (only mean values shown). Standard hypotonic solution with 48 mM sodium chloride (open circles) was used to elicit this current followed by 2 min perfusion in a solution containing 48 mM choline chloride (closed circles).

mM sodium chloride with sodium gluconate, reduced the outward current (decreased chloride influx) and shifted the reversal potential (Fig. 4 A[2]). Both current magnitude and reversal potential returned to their initial values after perfusion of the original hypotonic solution (Fig. 4 A[3]). The current-voltage relationship in Fig. 4 B
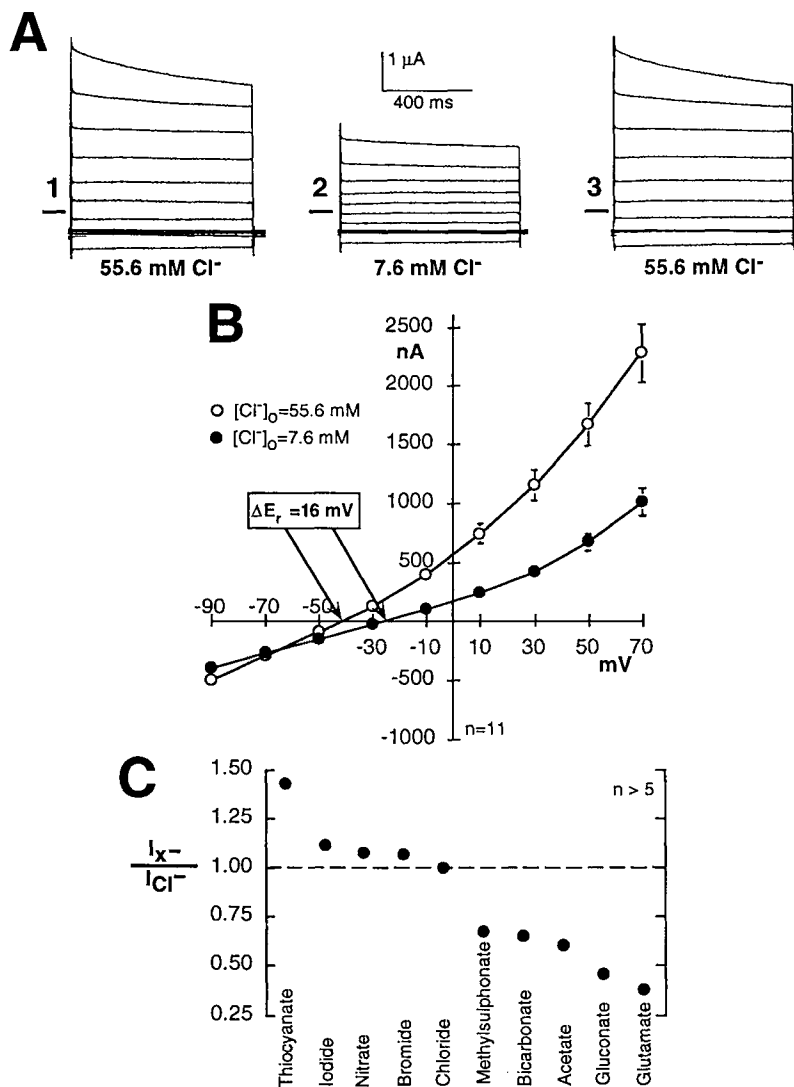
FIGURE 4.   Hypotonicity-activated current is a swelling-induced chloride current.

(*A*) Electrophysiological recording of oocyte during perfusion of hypotonic solution (1). Replacement of 48 mM sodium chloride with sodium gluconate shifted the reversal potential to the right and decreased the outward current (2). The initial profile was restored after perfusion of original hypotonic solution (3). Horizontal lines indicate zero current. Pulsing protocol used was the same as in Fig. 3.

(*B*) Current-voltage relationship of hypotonicity-activated current with 55.6 mM extracellular chloride (*open circles*) versus 7.6 mM chloride (*closed circles*). Sevenfold reduction in extracellular chloride shifted the reversal potential by 16 ± 1 mV and decreased the peak outward current from 2,276 ± 250 nA to 1,011 ± 120 nA at +70 mV (n = 11 oocytes).

(*C*) Relative anion conductivity through hypotonicity-activated current was determined by replacing 48 mM sodium chloride with test anion (five or more oocytes were measured for each anion tested). The anion conductivity sequence was $SCN^- > I^- \geq NO_3^- = Br^- > Cl^- >$ Methylsulphonate $\geq HCO_3^- \geq$ Acetate $>$ Gluconate $>$ Glutamate.
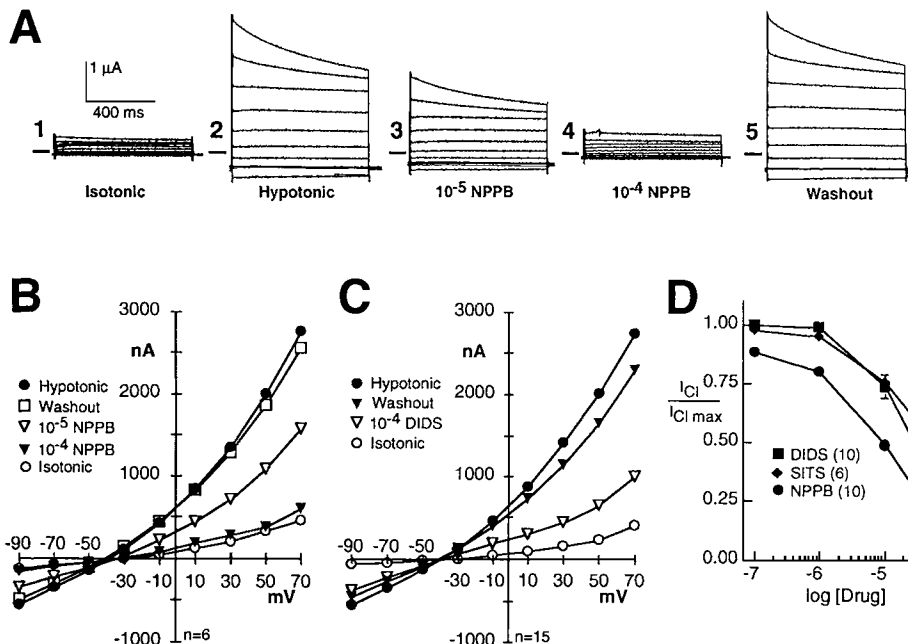
FIGURE 5. Hypotonicity-activated current is inhibited by chloride channel/transport blockers.

(A) Raw current traces demonstrating dose-dependent, voltage-independent, reversible block of swelling-induced chloride current by 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB). Background isotonic current (1) was first established using the pulsing protocol shown in Fig. 3. Perfusion of hypotonic solution (2) activated the endogenous current which was partially blocked by 10 μM NPPB (3). Addition of 100 μM NPPB (4) almost completely blocked the hypotonicity-activated current and was fully reversed after washout (5). Horizontal lines indicate zero current.

(B) Current-voltage relationship of the experiments shown in A (n = 6). Background isotonic currents (open circles) were −120 ± 39 nA and 452 ± 72 nA at −90 and +70 mV, respectively. Hypotonicity (closed circles) increased the current at −90 and +70 mV to −552 ± 69 nA and 2,764 ± 252 nA. 10 μM NPPB (open triangles) reduced the peak inward current to −352 ± 57 nA and peak outward current to 1,565 ± 193 nA. 100 μM NPPB (closed triangles) reduced $I_{Cl.swell}$ to −156 ± 54 nA and 604 ± 74 nA at −90 and +70 mV, respectively. The current was >90% recoverable on washout (open squares).

(C) Current-voltage relationship showing voltage-dependent, reversible sensitivity to DIDS (n = 15). Basal isotonic currents (open circles) were −66 ± 6 nA and 406 ± 37 nA at −90 mV and +70 mV increasing to −554 ± 39 nA and 2752 ± 267 nA after hypotonic perfusion (closed circles). 2 min after addition of 100 μM DIDS (open triangles), peak outward current was reduced to 1,003 ± 97 nA (>75%) while peak inward current was blocked to −381 ± 28 nA (35%). The peak outward current was restored 84% to 2,299 ± 235 nA after washout (closed triangles).

(D) Dose response curve of $I_{Cl.swell}$ for three known chloride channel blockers. After the hypotonicity-induced current reached its peak, various concentrations of drug were sequentially added to the bath to give final concentrations as shown. Current determinations were made after 90 s drug exposure by pulsing from V_h = −70 mV to +70 mV. At 100 μM final concentration, NPPB (closed circles) blocked $I_{Cl.swell}$ by 91 ± 3% (n = 10), DIDS (closed squares) 79 ± 2% (n = 10) and SITS (closed diamonds) 58 ± 4% (n = 6) at +70 mV. For IC_{50} values see Table I.

summarizes this dependence on extracellular chloride ($n = 11$). The sevenfold reduction in extracellular chloride (*closed circles*) had no effect on the magnitude of the inward current (outward Cl− flux), reduced the outward current (inward flux) from 2276 ± 250 nA (*open circles*) to 1,011 ± 120 nA at +70 mV (*closed circles*), and shifted the reversal potential by 16 mV.

Voltage-, cAMP-PKA- and swelling-activated chloride currents can be distinguished readily by their anion selectivity sequence. By replacing the 48 mM of sodium chloride in the standard hypotonic solution with various test sodium salts (such as sodium thiocyanate, sodium bromide, et cetera), the relative anion conductivity sequence for this endogenous current was estimated from the peak outward current (chloride influx) at +70 mV (Fig. 4 *C*). The relative peak currents achieved with the anion-substituted hypotonic solutions were 1.43 ± 0.04 (thiocyanate, $n = 6$), 1.11 ± 0.04 (iodide, $n = 5$), 1.08 ± 0.02 (nitrate, $n = 6$), 1.07 ± 0.01 (bromide, $n = 5$), 0.67 ± 0.03 (methylsulphonate, $n = 3$), 0.65 ± 0.02 (bicarbonate, $n = 6$), 0.61 ± 0.02 (acetate, $n = 7$), 0.45 ± 0.02 (gluconate, $n = 13$), and 0.38 ± 0.01 (glutamate, $n = 3$). For these experiments, the standard peak current was 2,467 ± 255 nA ($n = 21$). This sequence of conductivity was identical with that shown for swelling-induced chloride currents from various epithelial cells (McCann and Welsh, 1990). Relative permeabilities could not be determined because the oocyte's intracellular composition could not be controlled.

$I_{Cl.swell}$ *Is Blocked by Chloride Channel Blockers, Lanthanides, and Nucleotides*

$I_{Cl.swell}$ was reversibly blocked by a carboxylate analogue Cl− channel blocker, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) in a voltage-independent, dose-dependent manner (Fig. 5, *A*, *B*, and *D*). 10 μM NPPB decreased the peak outward current by 52% whereas 100 μM NPPB reduced the peak current by 91%. NPPB was equally effective in blocking the inward current (Fig. 5 *B*). The current returned to 92% of its original amplitude after washing out the NPPB. Two stilbene-derivative chloride channel blockers, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and 4-acetamido-4′-isothiocyanastilbene-2,2′-disulfonic acid (SITS), inhibited this chloride current in a voltage- and dose-dependent fashion. 100 μM DIDS (Fig. 5 *C*, $n = 15$) suppressed the peak outward current (75% block) to a greater extent than the peak inward current (35% block). 100 μM SITS blocked the outward current by 57% and the inward current by 25% ($n = 9$, data not shown). Both DIDS and SITS could be partially (85%) washed out. The dose response curve for the three blockers was determined at +70 mV (Fig. 5 *D*). The current was most sensitive to NPPB with an $IC_{50} = 10$ μM ($n = 10$, Table I). DIDS and SITS blocked $I_{Cl.swell}$ with IC_{50} values of 30 μM ($n = 10$) and 60 μM ($n = 6$) respectively (Fig. 5 *D*, Table I). Niflumic acid, a potent blocker of the calcium-activated chloride current, did not affect $I_{Cl.swell}$ at concentrations ≤50 μM (Table I).

The hypotonicity-activated chloride current was irreversibly suppressed by the trivalent cations, lanthanum, and gadolinium, in a dose-dependent fashion (Fig. 6, *A*, *B*, and *D*) with respective IC_{50} values of 60 μM ($n = 13$) and 250 μM ($n = 5$) (Table I). The background isotonic current (Fig. 6 *A*[1], *B*, $n = 5$) was 396 ± 21 nA which increased to 2,694 ± 270 nA at +70 mV after 5 min in hypotonic solution (Fig. 6 *A*[2], *B*). $I_{Cl.swell}$ was completely and irreversibly blocked to 370 ± 63 nA after being

exposed to 1 mM lanthanum for 90 s (Fig. 6A[4]). A 10 min washout (Fig. 6A[5]) increased the current to only 544 ± 133 nA. It is important to note that neither NPPB, DIDS, SITS, lanthanum nor gadolinium affected the basal current seen in isotonic conditions (Fig. 2A[1]). Barium had little effect on $I_{Cl.swell}$.

The endogenous $I_{Cl.swell}$ was sensitive to block by nucleotides. Extracellularly-applied cAMP attenuated the current with an IC_{50} value of 3.5 mM (n = 6, Fig. 6 D, Table I). The cAMP-mediated block was voltage-independent and reversible (Fig. 6 C). Addition of 1 mM membrane permeant dibutryl cAMP (n = 3) did not suppress $I_{Cl.swell}$ beyond that observed for cAMP suggesting an extracellular site of action. Similar concentrations (1–5 mM) of ATP and GTP partially blocked $I_{Cl.swell}$ as well (n = 4, data not shown).

TABLE I

Pharmacology of $I_{Cl.swell}$

Inhibitor	IC_{50}	
	μM	n
NPPB	10	10
DIDS	30	10
SITS	60	6
La^{+3}	60	13
Gd^{+3}	250	5
cAMP	3500	6

Summary of the effects of known chloride channel blockers and other compounds implicated in volume regulation on the hypotonicity-activated current in *Xenopus* oocytes. All compounds were added extracellularly and IC_{50} values were determined at +70 mV.

$I_{Cl.swell}$ and $I_{Cl.Ca}$ Represent Distinct Chloride Conduction Pathways

Chen et al. (1993) suggested that the calcium-activated chloride current ($I_{Cl.Ca}$) in oocytes increased twofold with hypotonicity. This was not observed during our investigation. First, we utilized the irreversible block by lanthanum to clearly distinguish $I_{Cl.swell}$ from $I_{Cl.Ca}$. $I_{Cl.swell}$ (Fig. 7A[1]) was irreversibly blocked by 1 mM lanthanum (Fig. 7A[2]). In the continued presence of lanthanum, the calcium ionophore A23187 (50 μM) triggered an outwardly rectifying, slowly activating current (τ_{on} = 242 ± 27 ms at +70 mV, n = 9) (Fig. 7A[3]). The current-voltage relationship summarized in Fig. 7 B shows $I_{Cl.swell}$ (*closed circles*) and the more steeply rectifying A23187-activated current (*closed squares*) elicited following irreversible block of $I_{Cl.swell}$ by lanthanum (*open diamonds*). The A23187-activated current could be blocked by NPPB (Fig. 7 C) and niflumic acid (data not shown), but not by lanthanum (Fig. 7 D). In fact, lanthanum and gadolinium (3–5 mM) activated the endogenous calcium-activated chloride current even in the absence of A23187 (data not shown), perhaps by a mechanism similar to that proposed for divalent cations (Miledi, Parker, and Woodward, 1989).

$I_{Cl.swell}$ was not noticeably affected by intracellular calcium. After incubating the oocytes in 50 μM BAPTA-AM for over 5 h to buffer the intracellular calcium, oocytes were voltage-clamped and exposed to hypotonic solution. Hypotonic exposure still
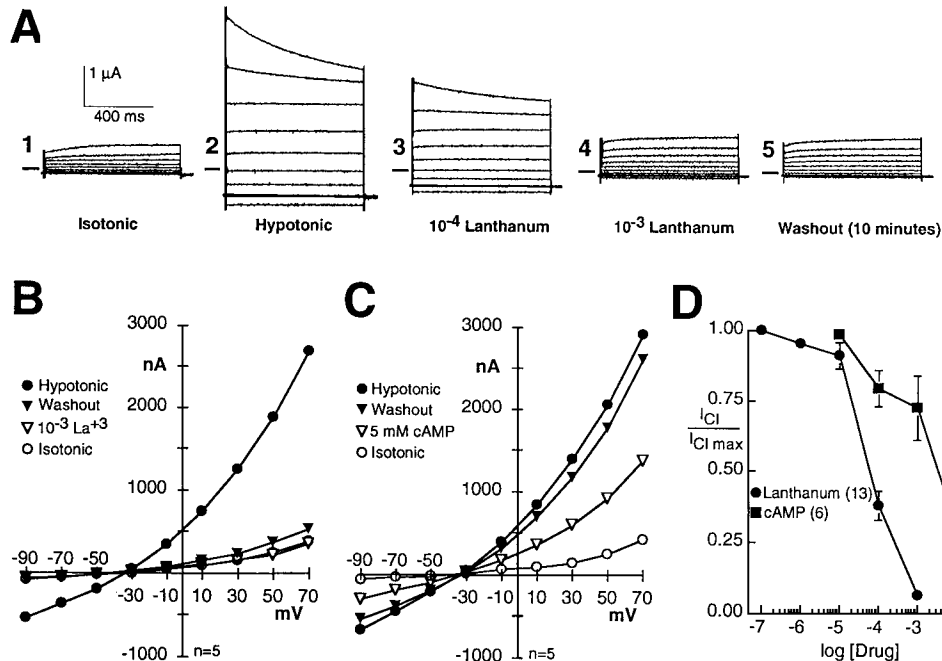
FIGURE 6. $I_{Cl.swell}$ is blocked by extracellular lanthanum and cAMP.

(*A*) Raw current traces demonstrating dose dependent, irreversible block of swelling-induced chloride current by lanthanum. Background isotonic current (1) was determined with pulsing protocol used in Fig. 3. Perfusion of hypotonic solution (2) activated $I_{Cl.swell}$ which was partially blocked by the addition of 100 μM lanthanum (3). Addition of 1 mM lanthanum (4) completely blocked the hypotonicity-activated current. Lanthanum block was irreversible (5) even after 15 min of washout. Horizontal lines indicate zero current.

(*B*) Current-voltage relationship demonstrating voltage-independence, irreversibility, and equipotent block by lanthanum (*n* = 5). Under isotonic conditions (*open circles*), the resting current was 396 ± 21 nA at +70 mV. Hypotonic exposure (*closed circles*) increased the peak outward current to 2,694 ± 270 nA. Within 90 s after the addition of 1 mM lanthanum (*open triangles*), the current was reduced to 370 ± 63 nA at +70 mV. Long periods of washout (> 10 min) (*closed triangles*) increased the outward current to 544 ± 133 nA.

(*C*) Current-voltage relationship showing voltage-independent sensitivity of $I_{Cl.swell}$ to cAMP (*n* = 5). Hypotonicity (*closed circles*) increased the current nearly sevenfold over resting isotonic conditions (*open circles*). 5 mM cAMP (*open triangles*) reduced $I_{Cl.swell}$ from 2,922 ± 315 nA to 1,375 ± 226 nA. Washout reversed 90% of the cAMP block (*closed triangles*).

(*D*) Dose response curve for $I_{Cl.swell}$ block by lanthanum and cAMP determined using a voltage step from −70 mV to +70 mV. 100 μM lanthanum (*closed circles*) blocked $I_{Cl.swell}$ by 62 ± 5%. 1 mM lanthanum blocked $I_{Cl.swell}$ by 94 ± 2% (*n* = 13). 10 mM cAMP (*closed squares*) reduced the current by 70 ± 4% at +70 mV (*n* = 6). For IC_{50} values see Table I.

elicited $I_{Cl.swell}$ (Fig. 8 *A*[1]) sensitive to lanthanum (Fig. 8 *A*[2]). However, subsequent addition of A23187 failed to stimulate the calcium-activated chloride current (Fig. 8 *A*[3]) suggesting that significant buffering of intracellular calcium occurred. In contrast to the paired untreated oocytes (Fig. 7 *B*, *closed squares*), A23187 was unable
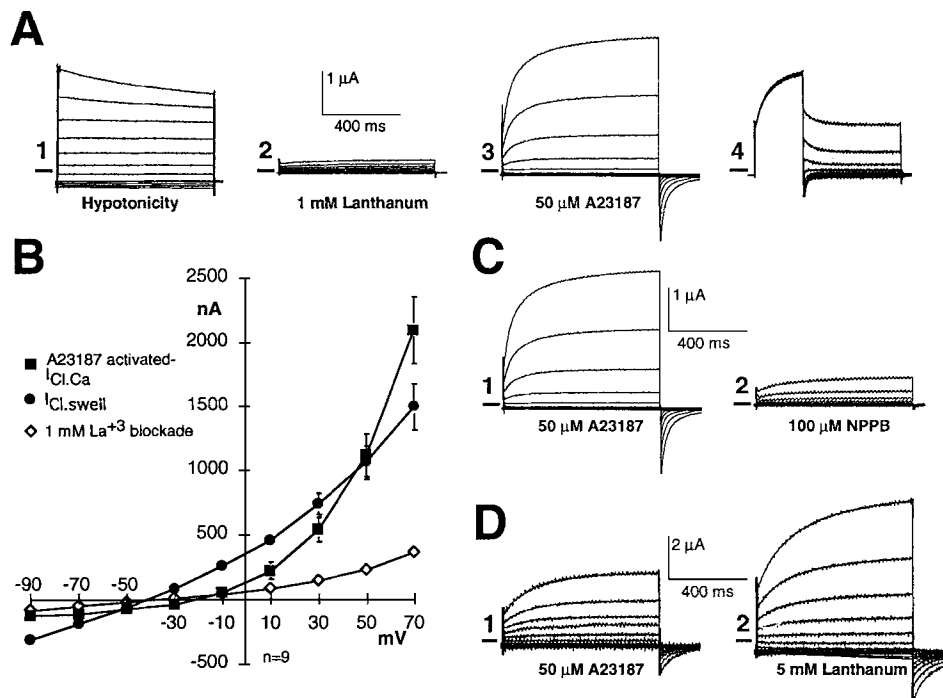
FIGURE 7. $I_{Cl.swell}$ is distinguished from the calcium-activated chloride current ($I_{Cl.Ca}$).

(A) Two electrode voltage clamp recording showing a rapidly activating (<1 ms), slowly inactivating current elicited by exposure to a hypotonic solution (1). After irreversible block of $I_{Cl.swell}$ by lanthanum (2), an outwardly rectifying, slowly activating (τ_{on} = 242 ± 27 ms; n = 9) calcium-activated chloride current was activated by 50 μM A23187 (3). Tail current measurements (4) demonstrate slowed deactivation and E_{rev} = −20 mV for this current. Horizontal lines indicate zero current. Voltage clamp profile was the same as in Fig. 3 (V_h = −70 mV).

(B) Current-voltage relationship for hypotonicity-induced chloride current (*closed circles*), irreversibly blocked $I_{Cl.swell}$ (*open diamonds*), and the calcium ionophore A23187 activated chloride current, $I_{Cl.Ca}$ (*closed squares*) (n = 9). In spite of irreversibly blocking the swelling-induced chloride current from 1,496 ± 180 nA to background levels of 366 ± 51 nA at +70 mV with 1 mM lanthanum, 50 μM A23187 activated an outwardly rectifying current to −130 ± 33 nA and 2,097 ± 262 nA at −90 and +70 mV, respectively.

(C) Raw current trace made under hypotonic conditions after blocking $I_{Cl.swell}$ with 1 mM lanthanum. A23187 activates the calcium-activated chloride current (1) which was blocked by 100 μM NPPB (2). Horizontal lines indicate zero current.

(D) A23187-stimulated current (1) was enhanced by 5 mM lanthanum (2).

to activate $I_{Cl.Ca}$ (Fig. 8 B, *open triangles*) in BAPTA-loaded oocytes. Nonetheless, $I_{Cl.swell}$ could still be activated by hypotonicity (Fig. 8 B, *open circles*). Likewise, the hypotonicity-activated chloride current displayed no requirement for extracellular calcium. $I_{Cl.swell}$ in oocytes (n = 6) bathed in a hypotonic solution containing 1.8 mM BaCl$_2$ instead of CaCl$_2$ as well as 0.2 mM EGTA responded identically to controls (Fig. 8 C). A summary of $I_{Cl.swell}$ and $I_{Cl.Ca}$ is shown in Fig. 9.
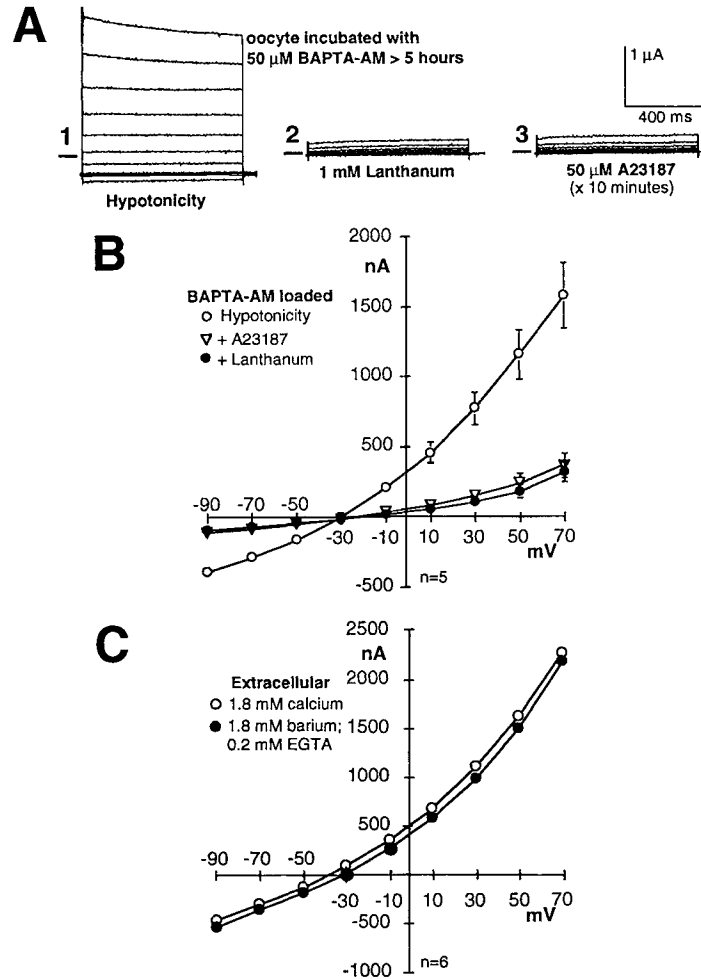
FIGURE 8. $I_{Cl.swell}$ does not require calcium.

(A) Raw current trace of oocyte previously incubated with 50 μM BAPTA-AM for over 5 h. Hypotonic perfusion (1) still activated the lanthanum-sensitive $I_{Cl.swell}$ (2). In contrast to paired oocytes from Fig. 7 A, subsequent addition of 50 μM A23187 (3) was not able to stimulate the calcium-activated chloride current, functionally demonstrating the intracellular buffering of calcium established by BAPTA-AM. Horizontal lines show position of zero current. Applied pulses were the same as in Fig. 3.

(B) Current-voltage relationship showing the ability of hypotonicity (*open circles*) to activate $I_{Cl.swell}$ following BAPTA-AM incubation (n = 5). Hypotonic solution (*open circles*) activated a current; −388 ± 38 nA and 1,575 ± 231 nA at −90 and +70 mV. 1 mM lanthanum completely inhibited $I_{Cl.swell}$ (*closed circles*) to −94 ± 36 nA and 315 ± 68 nA at −90 and +70 mV, respectively. Addition of calcium ionophore to BAPTA-AM loaded oocytes (*open triangles*) had little effect on the amplitude of the current in contrast to the paired oocytes in Fig. 7 B (*closed squares*).

(C) Current-voltage relationship comparing $I_{Cl.swell}$ with 1.8 mM Ca^{2+} present in the bath (*open circles*) versus a low calcium bath containing 1.8 mM Ba^{2+} and 0.2 mM EGTA (*closed circles*) (n = 6, only mean values are shown).
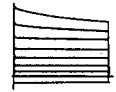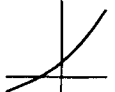
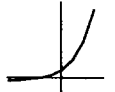| Chloride Current	Kinetics	I-V	Activated By Hypotonicity	Calcium Dependence	Inhibited By		
					NPPB	Niflumic Acid	La^{+3}
I$_{Cl.swell}$			+	-	++	-	+
I$_{Cl.Ca}$			-	+	+	+	-

FIGURE 9. I$_{Cl.swell}$ ≠ I$_{Cl.Ca}$. Summary of the electrophysiologic and pharmacologic properties that distinguish the hypotonicity-activated chloride current from the calcium-activated chloride current.

Inactivation Kinetics of I$_{Cl.swell}$ Are Modulated by Extracellular pH

Although pH is unlikely to serve as a general volume regulator, there is evidence that cellular pH may significantly modulate swelling-activated exchangers and channels (see Sarkadi and Parker, 1991). Extracellular pH modulated the kinetics of inactivation of I$_{Cl.swell}$. Exposure to the standard hypotonic solution at pH 7.5 evoked the current shown in Fig. 10 A(1). At pH 6.5 (Fig. 10 A[2]), the current inactivated more rapidly and displayed significant time-dependent inactivation at less depolarized potentials. Although the peak outward current was minimally affected (Fig. 10 B, left), the current in pH 6.5 inactivated at +70 mV with a time constant of 289 ± 10 ms (n = 14) compared with 461 ± 16 ms (n = 14) in pH 7.5 (Fig. 10 B, right). Switching to pH 8.5 (Fig. 10 A[3]) produced only a slight decrease in current (Fig. 10 B); 2,866 ± 234 nA at pH 6.5 (n = 12) versus 2,565 ± 220 nA at pH 8.5 (n = 12), but significantly lengthened time dependent inactivation at depolarized membrane potentials (τ = 659 ± 33 ms at +70 mV, Fig. 10 B).

I$_{Cl.swell}$: Mechanism of Activation

In our experiments, perfusion of a hypotonic solution activated a current which increased the membrane sensitivity to chloride (Figs. 1 B and 2). Given the composition of the hypotonic solution (see Materials and Methods), it is conceivable that either hypotonicity or a decrease in chloride concentration mediates the activation of I$_{Cl.swell}$ as both the osmolality and the concentration of sodium chloride were reduced by 50%. However, reducing the extracellular chloride concentration from 103.6 to 55.6 mM (replacing 48 mM of sodium chloride with sodium gluconate) while maintaining the same osmolality (220 mOsm) had a negligible effect on the oocyte's membrane potential (Fig. 1, A and C) and never activated I$_{Cl.swell}$ (n = 5, data not shown). On the other hand, exposure to a hypotonic solution having the same ionic strength as the isotonic solution (achieved by removal of sucrose) activated I$_{Cl.swell}$ (5/5, data not shown). Therefore, the activation of the chloride channels underlying I$_{Cl.swell}$ results from an osmotic gradient across the plasma membrane, rather than changes in chloride concentration.
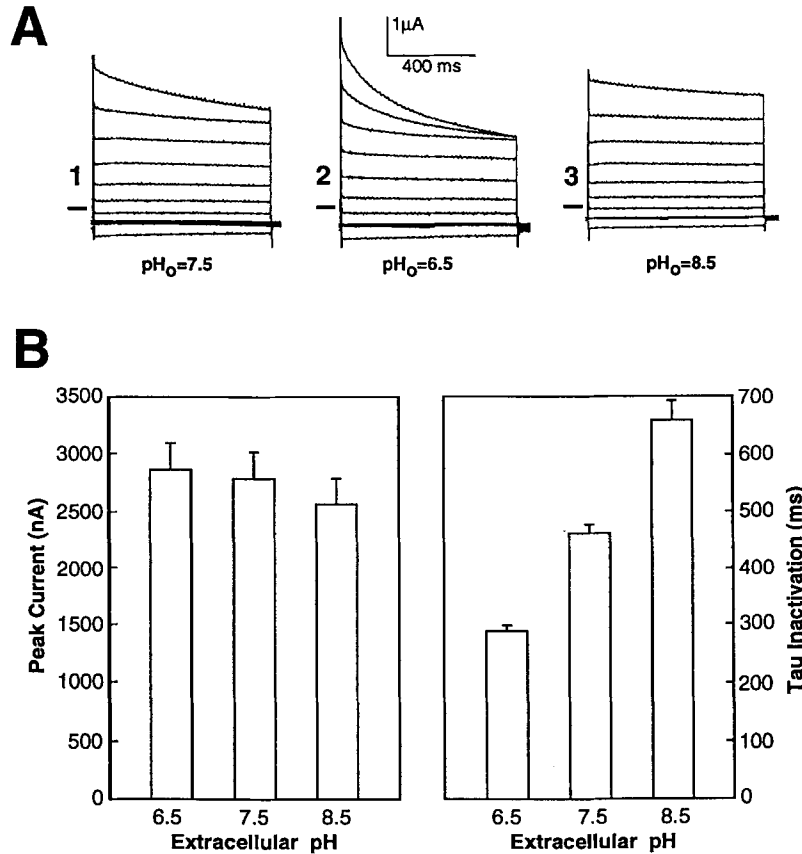
FIGURE 10. Inactivation kinetics of $I_{Cl.swell}$ depend on extracellular pH.

(*A*) Current was initially activated by standard hypotonic solution at pH 7.5 (1) followed by brief (<120 s) perfusion with an acidic (pH 6.5) hypotonic solution (2) followed with a basic (pH 8.5) hypotonic solution (3) using the same pulsing protocol as in Fig. 3. Inactivation of the swelling-induced current was accelerated at pH 6.5 and slowed at pH 8.5. Horizontal lines indicate zero current.

(*B*) Summary of the effects of extracellular pH on $I_{Cl.swell}$. Changing extracellular pH caused minor changes in the magnitude of peak current measured at 30 ms; 2,865 ± 234 nA at pH 6.5 to 2,565 ± 220 nA at pH 8.5 (*left; n* = 12). The time constant of inactivation (τ_{off}) determined at +70 mV changed substantially from 289 ± 10 ms to 461 ± 16 ms to 659 ± 33 ms at pH 6.5, 7.5, and 8.5, respectively (*right; n* = 14).

$I_{Cl.swell}$ varied over time after long exposure to the hypotonic solution and responded rapidly to changes in the osmotic gradient (Fig. 11). The two oocytes taken from different toads depicted in Fig. 11 reached their peak current approximately 5 min after hypotonic perfusion. One oocyte (*closed circles*) responded with a peak current of 1,500 nA that decreased slowly at a rate of 10 nA/minute in the continuous presence of hypotonic solution whereas the other oocyte (*open circles*)

responded with a peak current of 5,000 nA that decreased at a rate of 115 nA/min. The average rate of "rundown" was 59 ± 19 nA/min measured over 35 min ($n = 6$). Reperfusion of the isotonic solution produced a small (10%), transient (<1 min) increase in the amplitude of $I_{Cl,swell}$ perhaps secondary to the increased chloride concentration followed by an accelerated (>200 nA/min) rundown of the current. Subsequent perfusion of the hypotonic solution could once again activate $I_{Cl,swell}$ but to a lesser degree than the initial response.

G-protein, cAMP-PKA, and arachidonic acid-mediated signaling cascades do not affect $I_{Cl,swell}$. Injection of 100 μM GTPγS into oocytes failed to activate $I_{Cl,swell}$ ($n = 5$, data not shown) as did incubation with the membrane permeant dibutryl cAMP ($n = 3$ data not shown). Finally, 100 μM arachidonic acid ($n = 7$) failed to suppress and 2 μM leukotriene D_4 ($n = 2$) failed to activate $I_{Cl,swell}$ (data not shown).

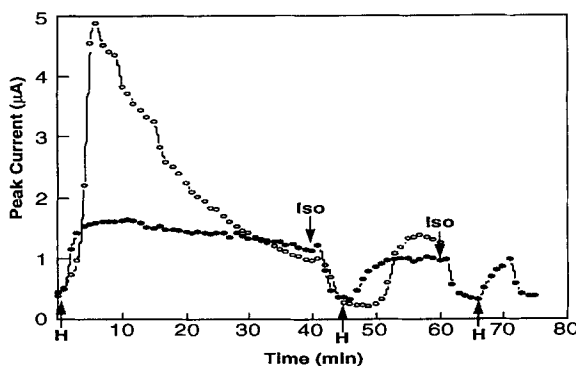The ability of the oocyte to activate $I_{Cl,swell}$ in response to a hypotonic environment

FIGURE 11. An osmotic gradient is responsible for activation of $I_{Cl,swell}$. These two oocytes represent the extremes in amplitude and kinetics observed for $I_{Cl,swell}$. Peak current values were determined every minute with an 800-ms pulse to +70 mV from $V_h = -70$ mV. Hypotonicity produced maximal activation within 5 min in both oocytes (open and closed circles). After 40 min in hypotonic solution, reperfusion of isotonic (Iso) solution caused a momentary increase in current amplitude (perhaps secondary to the increased chloride gradient) followed by a rapid decrease in current. Re-exposure to hypotonicity (H) was able to partially re-activate the chloride current. Returning to isotonic (Iso) perfusion once again inactivated $I_{Cl,swell}$.

diminished as a function of the time elapsed since defolliculation (Fig. 12 A). The amplitude of $I_{Cl,swell}$ was largest in freshly defolliculated oocytes (Fig. 12 A) and was virtually absent 6 d after defolliculation. At 12–24 h post-defolliculation, variability in $I_{Cl,swell}$ was apparent among oocytes from three different toads; 2,455 ± 286 nA (toad 1, $n = 8$), 3952 ± 308 nA (toad 2, $n = 10$), and 3,814 ± 678 nA (toad 3, $n = 7$). Oocytes from two of the three toads no longer elicited $I_{Cl,swell}$ in response to a hypotonic solution after 84–96 h (day 4). Substantial hypotonicity-activated currents (>2 μA) could be evoked in oocytes from toad 2 after day 4 ($n = 10$) becoming nonresponsive after day 6. This phenomena has been observed for the hyperpolarization-activated chloride current present in Xenopus oocytes as well (Parker and Miledi, 1988).

For $I_{Cl,swell}$, defolliculation rather than simple removal of the oocytes from the toad affected the activation. Oocytes that were surgically removed and defolliculated

immediately had no $I_{Cl.swell}$ after 6 d whereas the oocytes that were defolliculated after day 6 responded with peak currents averaging over 5 μA ($n = 5$, data not shown) when measured 4 h after defolliculation. Additionally, oocytes which were left enclosed in their follicular layer (Fig. 12 *B*[1]) responded to the hypotonic solution with substantially larger currents than that observed in oocytes that had been
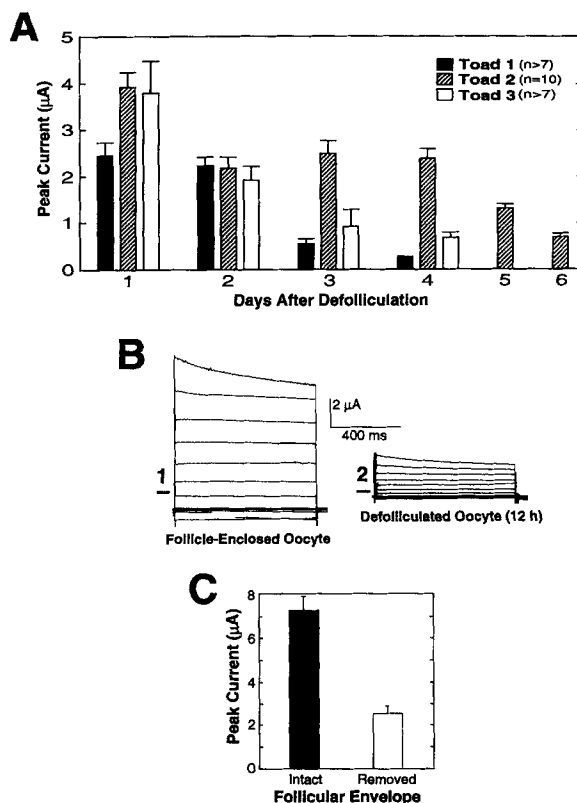

FIGURE 12. $I_{Cl.swell}$ diminishes as a function of time elapsed since defolliculation.

(*A*) Oocytes from three toads were defolliculated on day 0 and peak response to hypotonicity was measured at +70 mV 12–24 h later (day 1), 36–48 h later (day 2), 60–72 h later (day 3), et cetera. Numbers in parentheses indicate number of oocytes tested from each toad on each day after defolliculation. Day 1 measurements reveal variability in amplitude of $I_{Cl.swell}$: 2,455 ± 286 nA ($n = 8$), 3,952 ± 308 nA ($n = 10$), and 3,814 ± 678 nA ($n = 7$) for toad 1 (*closed*), 2 (*hatched*), and 3 (*open*). Oocytes from two of the three toads (*closed and open*) no longer responded to hypotonicity after 84–96 h (day 4). Oocytes from toad 2 (*hatched*) still responded with currents greater than 2 μA on day 4 decreasing to 676 ± 86 nA only by 132–144 h (day 6) after defolliculation.

(*B*) $I_{Cl.swell}$ in follicle-enclosed versus defolliculated oocytes. Electrophysiological recording of follicle-enclosed oocyte (1) and defolliculated oocyte (2) measured 12 h after defolliculation with same pulsing protocol as in Fig. 3. Horizontal lines indicate zero current.

(*C*) Summary of $I_{Cl.swell}$ elicited in follicle-enclosed oocytes and oocytes defolliculated 12 h earlier. Peak swelling-induced current was 7,276 ± 674 nA ($n = 7$) in follicle-enclosed oocytes (*solid bar*) compared with 2,545 ± 350 nA ($n = 18$) in defolliculated oocytes (*open bar*) from the same toad.

defolliculated 12 h earlier (Fig. 12 *B*[2]). The peak outward current in follicle-enclosed oocytes was 7,276 ± 674 nA ($n = 7$) compared to 2,545 ± 350 nA ($n = 18$) in defolliculated oocytes (Fig. 12 *C*). Exactly how the loss of the follicular envelope uncouples $I_{Cl.swell}$ from activation by swelling is uncertain, but a number of experimental approaches to examine this issue are ongoing.

DISCUSSION

$I_{Cl.swell}$ Defined

Xenopus oocytes exhibit a hypotonicity-activated, voltage-independent chloride conductance, $I_{Cl.swell}$, which is clearly distinct from nonselective mechanosensitive channels and calcium-activated chloride currents. $I_{Cl.swell}$ time-dependent inactivation was evident for depolarizations $> +30$ mV. Inactivation was substantially accelerated by decreasing extracellular pH. $I_{Cl.swell}$ is a chloride-conducting pathway as evidenced by its reversal potential approximating E_{Cl} in oocytes, dependence on extracellular chloride, and sensitivity to known chloride channel blockers.

$I_{Cl.swell}$ reversed at ~ -30 mV which is closer to the predicted chloride equilibrium potential, E_{Cl}, $(-13$ mV) than either E_K $(-96$ mV) or E_{Na} $(+51$ mV). A nonselective cation conductance, such as a stretch-activated channel, could conceivably have a similar reversal potential, but this seems less likely given the sensitivity of the current to the chloride channel blocker, NPPB. Equilibrium potentials were derived using intracellular ion concentrations determined by Barish (1983) in isotonic conditions. Barish (1983) found the intracellular chloride concentration to be ~ 33 mM but noted variability among toads. Exact determinations of E_{Cl} are complicated by the presence of undefined active exchange mechanism(s) which maintain intracellular chloride concentration (Dascal, 1987). How these transport systems behave under hypotonic conditions are similarly unknown. Conceivably, exposure to hypotonic solutions should decrease the concentration of intracellular chloride due to the 25% increase in volume and the increase in chloride efflux occurring during RVD. A reduction of $[Cl]_i$ from 33 mM to 17 mM would theoretically shift the E_{Cl} in the hypotonic solution from -13 mV to -30 mV.

The dependence of $I_{Cl.swell}$ on extracellular chloride concentration was clearly demonstrated (Fig. 4) although the change in reversal potential (16 mV) was less than the 50-mV shift predicted if the current was entirely chloride-selective. This discrepancy can be partially explained if gluconate is somewhat permeable through $I_{Cl.swell}$, thus attenuating the predicted shift. We found that using a hypotonic solution containing 48 mM sodium glutamate or 96 mM mannitol in place of sodium chloride, the reversal potential of $I_{Cl.swell}$ shifted by 25 mV $(n = 4)$. Even with this impermeant substitution, the shift in reversal potential was 50% of that predicted. If the intracellular concentration of chloride decreases during the perfusion of the low-chloride hypotonic solution, then the expected shift in reversal potential for a chloride-selective conductance might be substantially <50 mV. For example, the calculated shift would be only 25 mV if the intracellular chloride concentration decreased from 20 to 8 mM. The observation that the reversal potential of $I_{Cl.swell}$ becomes more hyperpolarized in the same hypotonic solution over time (data not shown) supports this hypothesis. Direct measurements of the intracellular chloride concentration in *Xenopus* oocytes during hypotonicity are needed to confirm this.

$I_{Cl.swell}$ displayed an anion conductivity sequence of $SCN^- > I^- \geq NO_3- = Br^- > Cl^- >$ Methylsulphonate $\geq HCO_3- \geq$ Acetate $>$ Gluconate $>$ Glutamate. This sequence corresponds with Eisenman's sequence I (Wright and Diamond, 1977) and suggests the osmosensitive conductance pathway contains weak binding sites for anions. $I_{Cl.swell}$ was reversibly blocked by two nonselective anion transporter antago-

nists, DIDS and SITS, and by the more selective chloride channel blocker, NPPB. Interestingly, $I_{Cl.swell}$ was sensitive to external trivalent lanthanides and nucleotides.

There was no significant contribution from SA-channels to $I_{Cl.swell}$ as evidenced by the negligible effect of extracellular cations, an estimated $P_{Cl}/P_{Na} > 3$, and sensitivity to chloride current blockers. Direct measurements of intracellular pressure in *Xenopus* oocytes exposed to a 60-mOsm hypotonic solution demonstrated that the intracellular pressure did not exceed 3 mm Hg (Kelly and Macklem, 1991). But, for comparison to patch recordings of stretch-activated channels, membrane tension, not pressure, must be measured in the membrane patch and whole oocyte. This data is not available. Given the oocyte's size, it is possible that the activation tension for SA-channels could actually be reached by relatively small changes in cell volume associated with small changes in internal pressure. Nonetheless, the hypotonic stimulus (110 mOsm) used in our experiments did not appear to elicit SA-channels to any significant degree because the current was entirely sensitive to chloride channel blockers.

$I_{Cl.swell}$ was clearly distinguished from the calcium-activated chloride current ($I_{Cl.Ca}$) in our study. $I_{Cl.swell}$ was not voltage-dependent, calcium-dependent, or sensitive to niflumic acid; characteristic features of $I_{Cl.Ca}$. We did not observe any potentiation of the calcium-activated chloride current by hypotonicity as was previously reported (Chen et al., 1993).

$I_{Cl.swell}$ and Other Swelling-induced Chloride Currents

The calcium independence and the insensitivity of the current to leukotrienes and arachidonic acid differentiates this current from the volume-sensitive chloride current involved in regulatory volume decrease in Ehrlich ascites tumor cells (Christensen and Hoffmann, 1992; Lambert, 1987). The chloride current detailed here, however, shares many of the same properties as volume-sensitive, swelling-induced chloride currents seen in human airway epithelial cells (McCann et al., 1989), a colonic tumor cell line (T84) (Worrell et al., 1989), human small intestinal epithelial cells (Kubo and Okada, 1992), ciliary ocular epithelial cells (Yantorno et al., 1992), cardiac myocytes (Tseng, 1992; Sorota, 1992), and lymphocytes (Cahalan and Lewis, 1988; Lewis, Ross, and Cahalan 1993). In each of these studies, reduction of the extracellular osmolality by 20–50% induced a chloride conductance. All the osmosensitive chloride currents were relatively voltage-independent (with the exception of the chloride current in T84 cells which activated at hyperpolarized potentials (Worrell et al., 1989)) with a similar time dependence of inactivation seen at depolarized membrane potentials. The anion conductivity sequence determined here coincided with that demonstrated by Kubo and Okada (1992), Lewis et al. (1993), McCann and Welsh (1990), and Worrell et al. (1989). This sequence ($I^- > Br^- > Cl^-$) is unlike that from the CFTR-mediated, cAMP-PKA activated chloride current ($Br^- > Cl^- > I^-$) (Anderson, Gregory, Thompson, Souza, Paul, Mulligan, Smith, and Welsh, 1991; Cliff and Frizzell, 1990) and the voltage-activated chloride currents ($Cl^- > Br^- > I^-$) (Jentsch et al., 1990; Steinmeyer, Ortland, and Jentsch, 1991). The measurable cation permeability observed is another common feature of several anion channels (Blatz and Magelby, 1985; Franciolini and Nonner, 1987; Strupp and Grafe, 1991; Welsh, 1986; Worrell et al., 1989).

Other swelling-induced chloride currents show comparable sensitivity to the carboxylate analogue chloride channel blocker (NPPB) (Kubo and Okada, 1992; Yantorno et al., 1992; Tseng, 1992) and to the stilbene-derivative channel blockers (DIDS and SITS) (Kubo and Okada, 1992; Doroshenko, Penner, and Neher, 1991; Lewis, Ross, and Calahan, 1993). Extracellular ATP has been shown to block reconstituted swelling-induced chloride channels from airway epithelial cells (Alton, Manning, Schlatter, Geddes, and Williams, 1991) and platelets (Manning and Williams, 1989), rectifying chloride channels in isolated membrane patches from airway epithelial cells (Stutts, Chinet, Mason, Fullton, Clarke, and Boucher, 1992), and the I_{Cln}-expressed chloride current (Paulmichl et al., 1992). Recently, a high affinity ATP derivative (trinitrophenyl-ATP) was shown to block ($IC_{50} = 0.27$ μM) reconstituted colonic chloride channels purported to mediate cell volume regulation (Venglarik, Singh, Wang, and Bridges, 1993). It will be interesting to see if other swelling-induced chloride currents share $I_{Cl.swell}$'s sensitivity to trivalent lanthanides. $I_{Cl.swell}$ closely resembles the volume-sensitive chloride current in small intestine epithelial cells (Kubo and Okada, 1992), differing primarily in its sensitivity to arachidonic acid. The epithelial chloride current was blocked by arachidonic acid ($IC_{50} = 8$ μM) (Kubo and Okada, 1992) whereas concentrations up to 100 μM had no effect on $I_{Cl.swell}$.

Intermediate conductance (25–75 pS) volume-sensitive chloride channels have been described in airway epithelia (Welsh, 1987; Solc and Wine, 1991), lobster walking leg nerves (Lukacs and Moczydlowski, 1990), T84 colonic cell line (Worrell et al., 1989), rat colonic epithelium (Diener et al., 1992), and Erhlich ascites cells (Hudson and Schultz, 1988). Small (2 pS) chloride channels in lymphocytes (Lewis et al., 1993) and large (400 pS) chloride channels from cardiac myocytes (Coulombe and Coraboeuf, 1992) have also been associated with volume regulation. The single channels in *Xenopus* oocytes underlying $I_{Cl.swell}$ have not yet been measured.

$I_{Cl.swell}$: Nature of the Osmotic Sensor

The precise mechanism of activation of swelling-induced chloride currents is unknown. This study in *Xenopus* oocytes and previous work in airway epithelial cells (McCann and Welsh, 1990) demonstrated that activation of chloride channels results from an osmotic gradient across the plasma membrane producing cell swelling. Although the volume expansion during hypotonicity was not measured in our study, similar reductions in osmolality have been shown to increase the volume in *Xenopus* oocytes by 25% (Kelly and Macklem, 1991). Our experiments indicate that no classical messenger systems appear to be involved in coupling swelling to the activation of $I_{Cl.swell}$. Calcium is not utilized as a second messenger for $I_{Cl.swell}$. This is similar to the calcium-independent chloride currents observed in airway epithelia (Kelly and Macklem, 1991), intestinal epithelia (Kubo and Okada, 1992), and cardiac myocytes (Tseng, 1992). Swelling does not appear to be transduced to $I_{Cl.swell}$ through G-proteins, cAMP-PKA, or lipophilic second messengers such as arachidonic acid or leukotriene D_4. This contrasts with the possible involvement of G-proteins in a chloride conductance in bovine chromaffin cells (Doroshenko et al., 1991; Doroshenko and Neher, 1992) and of leukotriene D_4 in Ehrlich ascites tumor cells (Christensen and Hoffmann, 1992; Lambert, 1987). It is uncertain what role the

cytoskeleton may play in the activation of $I_{Cl,swell}$. Agents which disrupt actin polymerization (cytochalasins) have been shown to both prevent and potentiate RVD (Hoffmann and Simonsen, 1989). However, the effects of cytochalasins on swelling-induced chloride currents have not been clearly established.

Membrane stretch or plasmalemmal tension generated during cell swelling could directly activate the channel protein or could be "sensed" by a cytoplasmic channel regulator. The observation that $I_{Cl,swell}$ decreased with the time elapsed since manual defolliculation is consistent with either possibility. Mechanical defolliculation could conceivably disrupt the synthesis of a key component in this transduction pathway since it is known that the endogenous rates of protein synthesis decrease following treatment of the oocyte in this manner (Smith, Xu, and Varnold, 1991). Alternatively, the removal of the follicular layer might render the plasma membrane more compliant over time and change the membrane forces giving rise to $I_{Cl,swell}$ activation.

The molecular identities of the channel protein and any involved "volume sensor" regulatory protein(s) are unknown. Three cloned proteins have been shown or suggested to participate in swelling-induced chloride conductances. First, we previously cloned a 235-amino acid protein (I_{Cln}) whose expression in oocytes gave rise to a nucleotide-sensitive chloride current (Paulmichl et al., 1992). I_{Cln} was cloned from Madin Darby canine kidney (MDCK) cells which effectively volume regulate after swelling (Paulmichl, Friedrich, Maly, and Lang, 1989; Weiss and Lang, 1992). The expressed I_{Cln}-protein-associated current resembled hypotonicity-activated chloride currents observed in T84 and cultured epithelial cells (Worrell et al., 1989; Okada and Hazama, 1989). The properties of I_{Cln} expressed in *Xenopus* oocytes (Paulmichl et al., 1992) are strikingly similar with the endogenous hypotonicity-induced chloride current ($I_{Cl,swell}$) described here. Both currents were calcium-independent, similarly suppressed by NPPB and DIDS, and comparably sensitive to nucleotides like cAMP and ATP. The key difference between $I_{Cl,swell}$ and expressed I_{Cln} is the manner in which they were elicited. In the previous work, I_{Cln} was rarely observed (3.9%) in control oocytes measured in isotonic media but was elicited in an isotonic (220 mOsm) bath in 65% of oocytes injected with cRNA encoding for the 235-amino acid protein (I_{Cln}) (Paulmichl et al., 1992). In the present study, the endogenous current $I_{Cl,swell}$ was observed in only 5 of 193 (2.6%) oocytes in the same isotonic solution but was nearly always seen (99%) after exposure to a hypotonic (110 mOsm) solution. Perhaps the overexpression of foreign I_{Cln} protein was able to bypass the requirement of reducing extracellular osmolality to activate the same endogenous population of chloride channels. We have recently discovered a protein homologous to I_{Cln} in *Xenopus* oocytes (Krapivinsky, Ackerman, Gordon, Krapivinsky, and Claptham, 1994). It remains to be determined whether this protein (I_{Cln}) is a link to the endogenous swelling-induced chloride conduction pathway rather than a channel itself.

Overexpression of P-glycoprotein, a 170-kD product of the multidrug resistance gene, in transfected NIH3T3 fibroblasts yielded a hypotonicity-activated chloride current (Valverde et al., 1992) similar to $I_{Cl,swell}$. Expressed P-glycoprotein was voltage-independent, time-dependent, and sensitive to NPPB and DIDS consistent with the properties of $I_{Cl,swell}$. While the expression of CFTR has been detected in *Xenopus* oocytes (Tucker, Tannahill, and Higgins, 1992), the presence of P-glycoprotein in *Xenopus* oocytes has not been established. 100 μM verapamil, which was shown

to inhibit the P-glycoprotein expressed current, did not suppress $I_{Cl.swell}$ ($n = 5$, data not shown) suggesting perhaps that a channel protein other than P-glycoprotein underlies $I_{Cl.swell}$. Overexpression of ClC-2 (907 amino-acids) in *Xenopus* oocytes was reported to induce a hyperpolarization-activated chloride current (Thiemann, Grunder, Pusch, and Jentsch, 1992) sensitive to extracellular hypotonic solutions (Grunder et al., 1992). However, the presence of both a hypotonicity-activated chloride current (present study) and a hyperpolarization-activated chloride current (Parker and Miledi, 1988) in *Xenopus* oocytes complicates the interpretation of ClC-2 expression.

Endogenous $I_{Cl.swell}$ must be accounted for if the effects of hypotonicity and swelling are to be determined for heterologously expressed proteins in *Xenopus* oocytes. The conclusions drawn previously that ClC-2 (Thiemann et al., 1992), I_{Cln} (Paulmichl et al., 1992), and phospholemman (Moorman et al., 1992) function as chloride channel proteins should be reexamined in light of the oocyte's endogenous chloride conductances. In fact, the role of phospholemman has recently been re-evaluated (Kowdley, Ackerman, John, Jones, and Moorman, 1993) and is now thought to be a possible modulator of the endogenous hyperpolarization-activated chloride channel. The recent assignment of CFTR as a water-permeable channel (Hasegawa et al., 1992) may also need to be reconsidered in light of oocyte $I_{Cl.swell}$. Because the oocyte becomes less capable of evoking its endogenous $I_{Cl.swell}$ in response to hypotonicity over time, heterologous expression studies performed at least 5 d after defolliculation may help minimize the contribution from native $I_{Cl.swell}$.

We have characterized a novel chloride current in *Xenopus* oocytes activated by extracellular hypotonicity that could participate in cell volume regulation. Swelling does not appear to elicit this current through common transduction cascades involving calcium, G-proteins, protein kinase A, or arachidonic acid metabolites. The molecular identities of the osmotic sensor, cytoplasmic modulators, and the chloride channel itself are yet to be determined. The presence of $I_{Cl.swell}$ in *Xenopus* oocytes provides an ideal cell system with which to probe the identities of these important molecules. $I_{Cl.swell}$ must be taken into account when assigning volume-regulatory/swelling-induced properties to foreign proteins expressed in *Xenopus* oocytes.

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