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Ca^{2+} SENSITIVITY OF VOLUME-REGULATORY K^+ AND Cl^- CHANNELS IN CULTURED HUMAN EPITHELIAL CELLS

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SUMMARY

1. During exposure to a hypotonic solution, cultured human epithelial cells (Intestine 407) exhibited a regulatory volume decrease (RVD) after initial osmotic swelling.

2. The volume readjustment was slowed by elevating the extracellular K^+ concentration and facilitated by reducing the extracellular Cl^- concentration. Not only putative K^+ channel blockers, quinine and Ba^{2+}, but also a stilbene derivative Cl^- channel blocker (SITS) inhibited the RVD.

3. The volume recovery of hypoosmotically swollen cells was very much suppressed by the deprivation of extracellular Ca^{2+} ions or by chelation of cytosolic Ca^{2+} ions with Quin-2 loaded within the cells.

4. Biphasic membrane potential changes were associated with the RVD process at low extracellular K^+ and Cl^- concentrations. The initial hyperpolarizing response was inhibited by quinine and Ba^{2+}, whereas the late depolarizing response was inhibited by SITS. The deprivation of extracellular Ca^{2+} inhibited the initial hyperpolarizing phase but not the late depolarizing phase.

5. Two-microelectrode voltage clamp studies showed that the initial hyperpolarization and late depolarization were associated with quinine-sensitive outward currents and SITS-sensitive inward currents, respectively. The reversal potentials estimated from the current–voltage curves were about -80 mV for the initial response and -27 mV for the late response. Tenfold changes in the K^+ and Cl^- concentrations shifted these reversal potentials by 50 mV for the initial response and by 42 mV for the late response.

6. Under whole-cell recordings, similar current changes were observed in the cells exposed to a hypotonic solution, when the intracellular Ca^{2+} ions were moderately buffered with 1 mM-EGTA in the dialysing solution filled in a patch pipette. When most Ca^{2+} ions were chelated with 10 mM-EGTA in the pipette solution, the initial outward current as well as the corresponding hyperpolarization was suppressed, but the late current associated with the depolarizing phase was preserved.

7. Intracellular Ca^{2+} injections induced an increase in the quinine-sensitive K^+ conductance but failed to activate the Cl^- conductance.

8. It is concluded that both K^+ and Cl^- channels are involved in the regulatory volume decrease, and that the former channel is exclusively activated by elevation of the cytosolic Ca^{2+} concentration in the epithelial cells.

INTRODUCTION

A variety of cell species are known to readjust their volume in anisotonic media after the initial osmotic volume changes (see Kregenow, 1981; Spring & Ericson, 1982; Grinstein, Rothstein, Sarkadi & Gelfand, 1984, for reviews). The regulatory volume decrease (RVD) following osmotic swelling mainly results from the loss of KCl during the exposure to a hypotonic solution. Thus, conductive K^+ and Cl^- pathways have been suggested to be responsible for the RVD mechanism in a number of mammalian cell species (Grinstein, Clarke, Dupre & Rothstein, 1982*a*; Hoffmann, Simonsen & Lambert, 1984; Sarkadi, Attisano, Grinstein, Buchwald & Rothstein, 1984*a*). However, the presence of such separate K^+ and Cl^- currents in the RVD process has not as yet been demonstrated. Also, electroneutral co-transport pathways including a K^+–Cl^- symporter or K^+–H^+ and Cl^-–HCO_3^- antiporters have been shown to be activated during the RVD in some cells (Schmidt & McManus, 1977; Cala, 1980; Dunham & Ellory, 1981; Kregenow, 1981; Parker, 1983; Larson & Spring, 1984; Lauf, 1985). By means of microelectrodes, hyperpolarizations have been observed upon exposure to a hypotonic solution (Kleinzeller, Nedvidkova & Knothova, 1967; Armstrong, Byrd, Cohen, Cohen, Hamang & Myers, 1975; Lau, Hudson & Schultz, 1984; Reuss, 1985; Costa, Fernandes, Ferreira, Ferreira & Giraldez, 1987; Roy & Sauvé, 1987), but the ionic mechanism and its crucial role in the volume regulation were not investigated under voltage clamp.

Recently, it has been shown that cytosolic Ca^{2+} ions are elevated by a hypotonic challenge (Cala, Mandel & Murphy, 1986; Wong & Chase, 1986). Several lines of circumstantial evidence have suggested that activation of volume-regulatory K^+ transport systems is controlled by cytosolic Ca^{2+} ions (Grinstein, Dupre & Rothstein, 1982*b*; Cala, 1983; Hoffmann *et al.* 1984; Foskett & Spring, 1985; Sarkadi, Cheung, Mack, Grinstein, Gelfand & Rothstein, 1985; Davis & Finn, 1987). Hoffmann, Lambert & Simonsen (1986) have raised the possibility that cytosolic Ca^{2+} ions are also involved in the activation of volume-regulatory Cl^- channels during the RVD since activation of Cl^- transport is induced not only during the RVD but also upon A23187-induced shrinkage of Ehrlich ascites tumour cells. In human lymphocytes, however, the RVD process has been shown to take place even in Ca^{2+}-depleted cells in which high cationic conductances had been ensured by the application of gramicidin (Grinstein *et al.* 1982*b*). Thus, the Ca^{2+} dependency of volume-regulatory ion channels is not entirely certain at present.

In the present study, the conductive properties and Ca^{2+} sensitivity of KCl transport pathways responsible for the RVD process in cultured human epithelial (Intestine 407) cells were investigated by electrophysiological approaches, including conventional intracellular recordings, two-microelectrode voltage clamp studies and tight-seal whole-cell recordings. The results show the independent operations of Ca^{2+}-activated K^+ channels and Ca^{2+}-insensitive Cl^- channels during the RVD process.

Preliminary accounts of some of these results have been given in abstract form (Hazama & Okada, 1987*a*, *b*).

METHODS

Cells. Uncloned Intestine 407 cells derived from human embryonic intestine were purchased (Flow Labs Inc.) and were cultured in Fischer medium supplemented with 10% newborn calf serum (Flow Labs Inc.) on plastic dishes (3 or 5 ml). The monolayer of giant Intestine 407 cells (containing several to tens of nuclei) was obtained by cell fusion with polyethylene glycol (PEG), as described previously (Yada & Okada, 1984), for electrophysiological studies. The enlarged cell size could minimize leakage artifacts due to penetration of microelectrodes. The monolayer of small Intestine 407 cells (without PEG treatment) was also provided for whole-cell recordings. For measurements of the mean cell volume, the cells in suspension at a density of about 5×10^6 cells/ml were prepared by detaching from plastic substrates with dispase (500 units/ml) and cultured with agitation for more than 3 h, during which the suspended cells could recover from the transient permeability perturbation caused by detachment and enzyme treatment (Lamb & Ogden, 1987). Quin-2-loaded cells in suspension were prepared by incubating with 50 μM-Quin-2/AM (Dotite) and serum for 15 min at 37 °C. Cell viability determined by the Trypan Blue exclusion test was > 90% and was not significantly affected by the anisotonic challenge.

Cell volume measurements. The mean cell volume (MCV) of Intestine 407 was measured by an electrical sizing technique using a Coulter counter (Toa Medical Electronics Co., CC-150A) adapted with a 150 μm diameter aperture. The time course of the MCV changes was automatically measured by a microcomputer connected to the Coulter counter via an amplifier, a peak-holder circuit and an analog-to-digital (A/D) converter, as reported previously (McGann, Turner & Turc, 1982).

Peak amplitudes of the signal output from the Coulter counter, which are known to be usually proportional to the cell volume (Gregg & Steidley, 1965), were maintained for 200 μs by a peak-holder circuit. The mean peak amplitudes of the signals for 5 s (via an A/D converter) were calculated (by a computer) every 20 s. To calibrate the MCV values, polystyrene latex beads (9·6, 14·6 and 19·1 μm in diameter, Duke Scientific Corporation) were used as standards. The instrumental readings were dependent on the ionic strength of the suspending medium, but were linearly related to the volume at a given ionic strength (Fig. 1*A*). Figure 1*B* shows a representative cell volume distribution of Intestine 407 cells suspended in an isotonic medium. The distribution curve usually showed a major peak and additional small peaks at about two and three times the volume of the major peak due to cell aggregation. Since the distribution of the major peak was approximately Gaussian, this peak value was taken as the MCV value.

Anisotonic challenges were performed by exposing the cell suspended in an isotonic saline solution to a fiftyfold volume of a hypotonic or hypertonic saline solution.

Electrophysiology. Changes in the membrane potential in response to a hypotonic challenge were measured with microelectrodes filled with 3 M-KCl (resistance, 30–100 MΩ; tip potential, less than 5 mV). The procedures for conventional recordings were essentially the same as those described previously (Okada, Tsuchiya, Yada, Yano & Yawo, 1981). The input (membrane) resistance was monitored by passing constant-current pulses of 0·3 nA through the recording electrode via a bridge circuit (WPI, KS-700). Intracellular Ca^{2+} injections were made by applying outward currents of 5 or 10 nA through an additional microelectrode, filled with 0·1 M-$CaCl_2$ and 0·5 M-KCl, impaled simultaneously.

Changes in the membrane current in response to a hypotonic challenge were measured at a certain holding potential by a two-microelectrode voltage clamp method with an amplifier (Nihonkohden Kogyo Co., CEZ-1100). Current measurements at different holding potentials were made in different cells, because the rate of RVD and the pattern of electrical responses were both altered if the cell were hypotonically challenged more than once. Voltage clamping was intermittently released to monitor the zero-current membrane potential profile and to avoid intense changes in the cellular ionic composition.

Whole-cell recordings with tight-seal patch electrodes (tip bore, about 1 μm) were performed, as described by Hamil, Marty, Neher, Sakmann & Sigworth (1981) with an amplifier (Nihonkohden Kogyo, S-3666). Measurements of membrane currents at certain holding potentials and zero-current membrane potentials were alternately made in relatively small PEG-fused cells containing several nuclei. Normal cells without PEG treatment were also employed in several whole-cell recordings, though the absolute value of zero-current potentials was often found to be inaccurate

because of the high intrinsic resistance of small Intestine 407 cells and resultant large leak currents from the patch-electrode seal.

Electrophysiological experiments during the process of volume regulation were carried out by changing the perfusate from an isotonic to an anisotonic saline solution at a rate of about 30 ml/min.

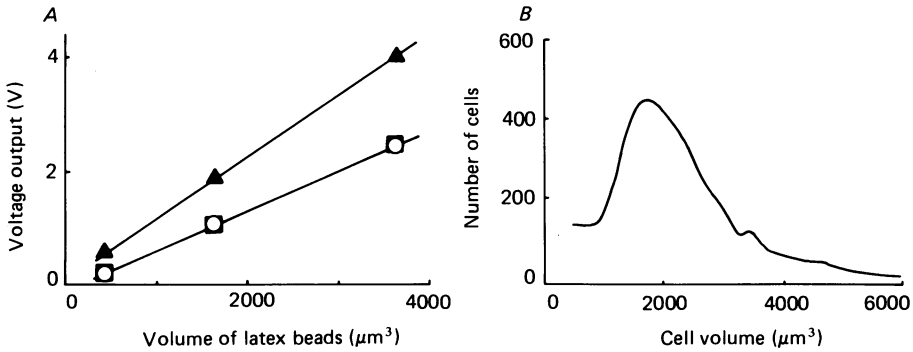

Fig. 1. Volume calibration with latex beads and volume distribution of Intestine 407 cells. *A*, the relationship between peak amplitudes of the signal output from the Coulter counter and the volumes of standard latex beads suspended in the control isotonic (○), hypotonic (0·55 × isotonic, ▲) and hypertonic (1·33 × isotonic, ■) saline solutions. The ionic strength of an isotonic saline solution was different from that of a hypotonic saline solution, but was identical to that of a hypertonic saline solution (for their compositions: see text). *B*, representative volume distribution of Intestine 407 cells suspended in the control isotonic saline solution.

Media and chemicals. The control isotonic or hypotonic saline solution had the following composition (in mM): NaCl, 137·5 or 54; KCl, 4·2; $CaCl_2$, 0·9; $MgCl_2$, 0·5; mannitol, 20 or 26·6; Na-HEPES, 6; and HEPES, 8 (pH 7·4; osmolarity measured by an osmometer, 293 or 161 mOsM). The control hypertonic saline solution was prepared by adding 100 mM-mannitol to the control isotonic saline solution. An isotonic saline solution in which 150 mM-mannitol was added to a control hypotonic saline solution was also employed in some experiments. A high-K^+ (42 mM) isotonic or hypotonic saline solution was made by replacing 37·8 mM-NaCl in the control saline with KCl. A low-Cl^- (6 mM) isotonic or hypotonic saline solution was made by replacing 50·8 mM-NaCl in the control saline solution with 25·4 mM-Na_2SO_4 and 27 mM-mannitol, and a low-Na^+ (6 mM) or Na^+-free saline solution was made by replacing NaCl by Tris–HCl. A Ca^{2+}-free isotonic or hypotonic saline solution was prepared by adding 1 mM-ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, Nakarai Chemical Co.) to the control saline solution devoid of $CaCl_2$. For whole-cell recordings, the patch electrode was filled with a solution composed of 73·5 mM-KCl, 73·5 mM-potassium aspartate, 2 mM-$MgCl_2$, 1 mM-ATP, 10 mM-Na-HEPES and 10 mM-HEPES (pH 7·3). The free Ca^{2+} concentration was adjusted to pCa > 10 by adding 10 mM-EGTA or to pCa 6·75 by adding 1 mM-EGTA as well as an appropriate amount of $CaCl_2$ to the solution.

Quinine HCl, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS) and frusemide were purchased from Nakarai Chemical Co.

All experiments were performed at room temperature (24–26 °C).

RESULTS

Regulatory volume decrease

The mean volume of Intestine 407 cells suspended in the saline solution without any nutrients and serum remained unchanged for over 30 min under isotonic conditions (Fig. 2*A*). When the cells were exposed to a control hypotonic saline solution of 55% osmolarity, they rapidly underwent an initial swelling, as expected.

This initial increase in volume was followed by a spontaneous recovery to near-control levels within 2–3 min, despite the continued exposure to the hypotonic bathing solution (Fig. 2*B*). The cells suspended in an isotonic saline solution containing 150 mM-mannitol showed the same volume response when tonicity of the bathing solution was decreased by removing mannitol while keeping the electrolyte

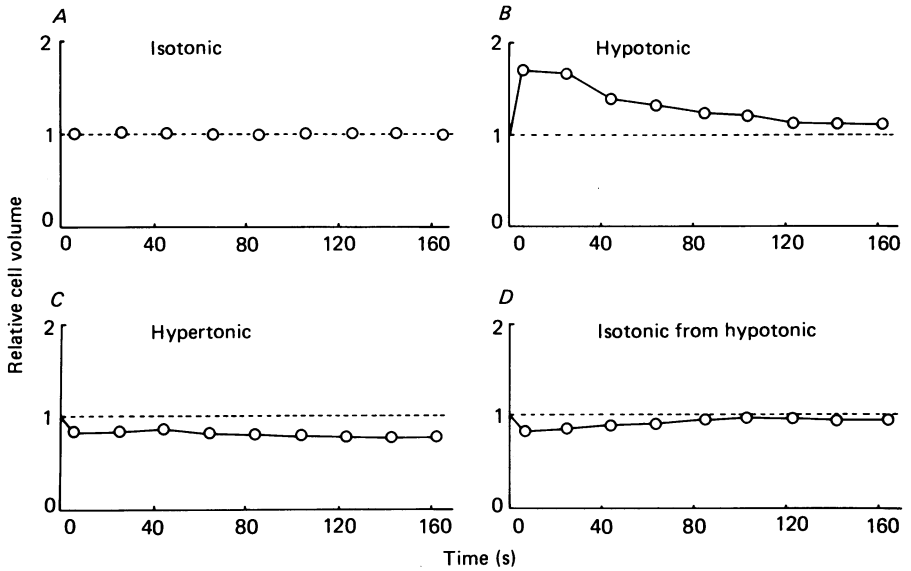

Fig. 2. Time course of volume changes in Intestine 407 cells in response to a variety of osmotic challenges. At zero time, the cells suspended in the control isotonic saline solution were exposed to the control isotonic (*A*), hypotonic (0·55 × isotonic, *B*) or hypertonic (1·33 × isotonic, *C*) saline solution. In *D*, the cells equilibrated in the control hypotonic saline solution were exposed to the control isotonic saline solution.

composition constant. Thus, Intestine 407 cells can exhibit a regulatory volume decrease, as found in many other cell species (Kregenow, 1981; Spring & Ericson, 1982; Grinstein *et al.* 1984). Fused giant Intestine 407 cells produced by PEG treatment also showed a regulatory volume decrease in response to a hypotonic challenge, though the rate of volume recovery was slower than normal cells (data not shown).

When the cells were exposed to a hypertonic saline solution of 133% osmolarity, cell shrinkage was instantaneously induced and remained stable for over 30 min (Fig. 2*C*) without exhibiting a regulatory volume increase (RVI). The RVI was, however, observed when an isotonic medium was applied to the cells which had been equilibrated with a hypotonic saline solution (Fig. 2*D*), as found in other cell species (Roti Roti & Rothstein, 1973; Grinstein, Clarke & Rothstein, 1983; Hoffmann, Sjoholm & Simonsen, 1983; Roy & Sauvé, 1987). This immediate shrinkage in response to an osmotic challenge with an isotonic saline solution implies that net reduction of intracellular osmoles occurred after attaining volume regulation under hypotonic conditions.

Elevation of the K^+ concentration in the bathing solution from 4·2 to 42 mM inhibited the volume recovery of hypotonically swollen cells (Fig. 3*A*; ○). In

contrast, a reduction of the Cl^- concentration from 60 to 6 mM facilitated the RVD process (Fig. 3*B*; △). A decrease in the Na^+ concentration from 60 to 6 mM had no significant effect on the time course of the RVD (data not shown). Quinine, which is known to block K^+ conductances in epithelial cells (Hazama, Yada & Okada, 1985; Yada, Oiki, Ueda & Okada, 1986) suppressed the RVD process (Fig. 3*A*; ●).

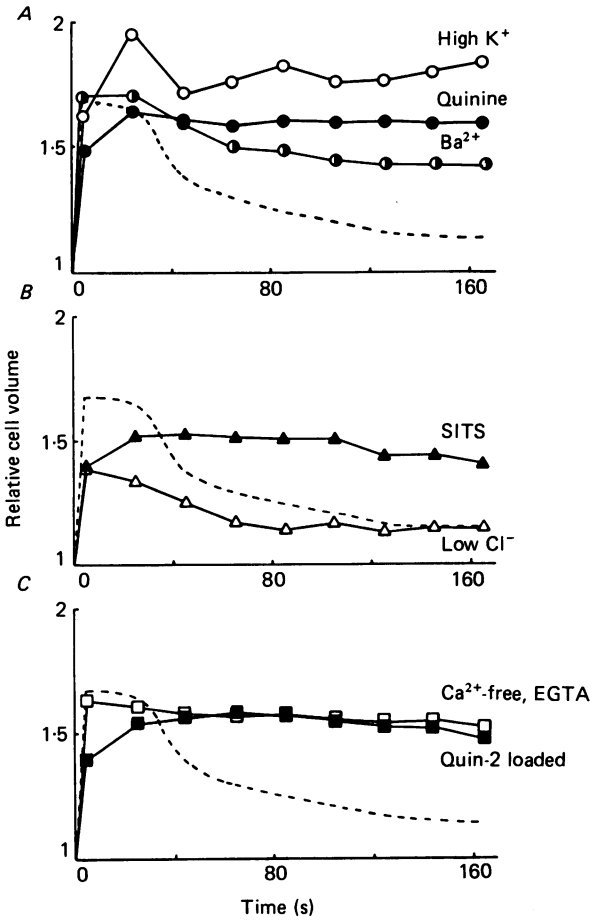

Fig. 3. Effects of treatment with ion channel blockers and of changes in the ionic environments on the regulatory volume decrease in Intestine 407 cells. At zero time, the cells were subjected to a hypotonic challenge. ○, 42 mM-K^+; ●, 0·4 mM-quinine (prior treated for 5 min); ◑, 1 mM-Ba^{2+} (5 min); △, 6 mM-Cl^-; ▲, 0·1 mM-SITS (5 min); □, 1 mM-EGTA in Ca^{2+}-free solution; ■, Quin-2 loading (see Methods); dashed line, control (4·2 mM-K^+, 60 mM-Cl^-) reproduced from Fig. 2*B*.

Ba^{2+}, another epithelial K^+ channel blocker (Nagel, 1979; Kirk, Halm & Dawson, 1980; Hunter, Lopes, Boulpaep & Giebisch, 1984; Wills, 1985; Gitter, Beyenbach, Christine, Gross, Minuth & Fröemter, 1987) similarly suppressed the RVD (Fig. 3*A*; ◑). A stilbene derivative inhibitor of Cl^- channel, SITS (White & Miller, 1979; Nelson, Tang & Palmer, 1984) also inhibited the RVD (Fig. 3*B*; ▲). However, the RVD was not inhibited by frusemide (0·1–1 mM; data not shown), which is a powerful inhibitor of Na^+–Cl^- and Na^+–K^+–$2Cl^-$ co-transport (Humphreys, 1976;

Palfrey, Silva & Epstein, 1984; Geck & Heinz, 1986) and is also known to block Ca^{2+}-activated Cl^- conductance in epithelial cells (Evans, Marty, Tan & Trautmann, 1986). These data indicate that the KCl efflux, presumably via K^+ and Cl^- channels, is a prerequisite to the RVD in Intestine 407 cells.

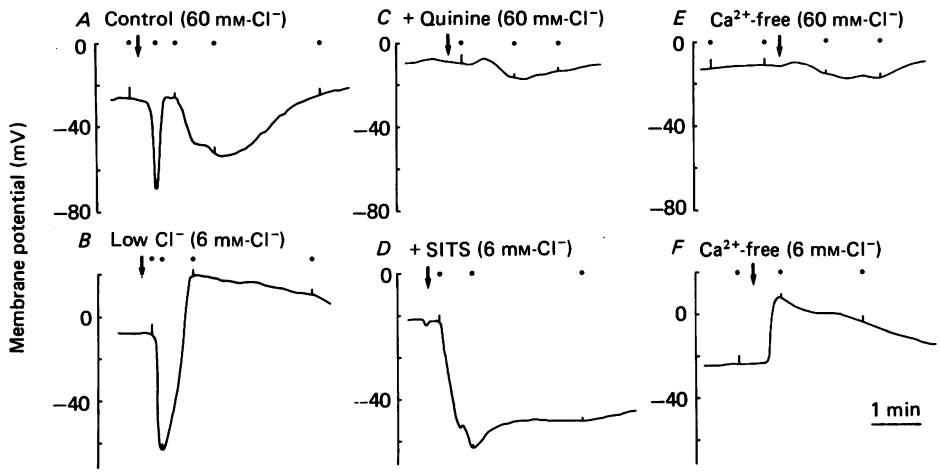

Fig. 4. Changes in the membrane potential in the Intestine 407 cells after exposure to a hypotonic solution (55% osmolarity at arrows) under a variety of experimental conditions. *A*, *C* and *E*, at control monovalent ion concentrations (60 mM-Cl^-, 4·2 mM-K^+, 60 mM-Na^+). *B*, *D* and *F*, at low Cl^- (6 mM-Cl^-). *E* and *F*, at low Ca^{2+} (1 mM-EGTA, no added Ca^{2+}). Quinine (0·4 mM, *C*) or SITS (0·1 mM, *D*) was added 5–10 min before the hypotonic challenge. The input (membrane) resistance was monitored by passing constant-current pulses of 0·3 nA (at dots) through the recording electrode with a bridge circuit.

TABLE 1. Effects of ion concentrations on the electrical potential changes in response to a hypotonic challenge

	Concentration (mM)				Mean membrane potential ± S.E.M. (mV)		
	K^+	Na^+	Cl^-	n*	Resting	First phase	Second phase
Control	4·2	60	60	5	−20·7 ± 2·9	−69·8 ± 2·0	−30·6 ± 1·3
High K^+	42	60	60	5	−13·0 ± 2·0	−27·6 ± 3·3†	−13·4 ± 3·6†
Low Cl^-	4·2	60	6	3	−8·3 ± 0·3†	−51·6 ± 7·3†	+15·7 ± 2·9†
Na^+-free	4·2	0	60	4	−15·6 ± 3·0	−58·6 ± 4·9	−37·4 ± 4·2

* Number of observations.
† Significantly differs at $P < 0·05$ from the control value.

The deprivation of extracellular Ca^{2+} ions with 1 mM-EGTA slowed the RVD process (Fig. 3*C*; □). A similar effect was also observed in Quin-2-loaded cells (Fig. 3*C*; ■), in which cytosolic Ca^{2+} ions would be chelated and clamped at a fixed concentration (Tsien, 1981; Knight & Kesteven, 1983; Elferink & Deierkauf, 1985; Wong & Chase, 1986). Therefore, it is concluded that the RVD process is highly sensitive to cytosolic Ca^{2+} ions. This raises a possibility that cytosolic Ca^{2+} ions may be linked to volume-regulatory K^+ and Cl^- channels.

Membrane potential changes associated with the regulatory volume decrease

Giant Intestine 407 cells produced by PEG-mediated cell fusion showed biphasic hyperpolarizations interposed by a transient depolarization (second phase) in

response to a reduction in osmolarity to the perfusate under the control electrolyte conditions (4·2 mM-K^+, 60 mM-Na^+, 60 mM-Cl^-) (Fig. 4*A*, Table 1). During intracellular recordings upon each hypotonic challenge, cell swelling and subsequent volume recovery were always observed under a phase-contrast microscope. The changes in the membrane potential (including the second-phase depolarization) were

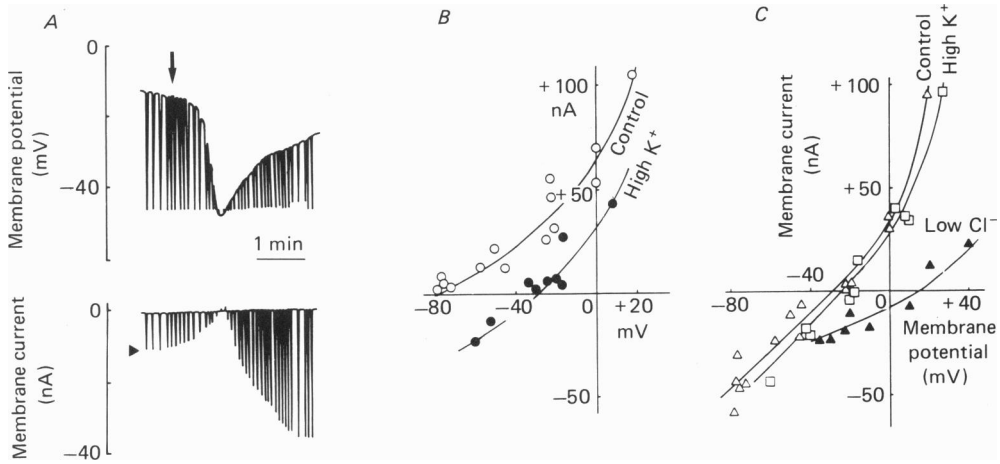

Fig. 5. Changes in the membrane current in response to a hypotonic challenge under a two-microelectrode voltage clamp. *A*, representative records of changes in the membrane potential (upper trace) and the membrane current (lower trace) after a hypotonic challenge (55% at an arrow). Voltage clamping was intermittently released to monitor the zero-current membrane potential and to avoid intense perturbation in the intracellular ionic milieu. The resting current level is indicated by a arrow-head. Inward currents were expressed as downward deflections. *B*, current–voltage relations for the first-phase (hyperpolarizing) response under the control electrolyte conditions (4·2 mM-K^+, 60 mM-Cl^-, ○) and high-K^+ conditions (42 mM-K^+, 60 mM-Cl^- ●). *C*, current–voltage relations for the second-phase (depolarizing) response under the control electrolyte conditions (△), high-K^+ conditions (□) and low-Cl^- conditions (6 mM-Cl^-, 4·2 mM-K^+, ▲). Each symbol represents the current change from the resting level to the peak response. Currents were observed at different holding potentials in different cells. Relatively large giant cells with a comparable cell size (containing fifteen to twenty nuclei) were selected to minimize the variability in the membrane current magnitude.

associated with a decrease in the input resistance (Fig. 4*A*, at dots), suggesting increased membrane conductance to some ions. The first-phase hyperpolarization was markedly diminished (by about 42 mV) by elevation of the K^+ concentration (to 42 mM) in the perfusate (Table 1), whereas this response was less affected by a reduction in extracellular Cl^- (Fig. 4*B*, Table 1) or by the deprivation of Na^+ (Table 1). These results indicate that the first-phase hyperpolarization is produced primarily by an increase in the K^+ conductance. In contrast, the depolarization phase was remarkably affected by a reduction in the extracellular Cl^- concentration. At 6 mM-Cl^-, the initial hyperpolarization was followed by a marked positive-going potential change, showing an overshoot by about 16 mV during the exposure to a hypotonic solution (Fig. 4*B*, Table 1). The second-phase potential was, to a lesser extent, affected by elevation of the extracellular K^+ level or by the deprivation of Na^+ (Table 1). These results show that an increase in the Cl^- permeability is responsible for the

second-phase electrical response to a hypotonic challenge, though some contribution of the Na^+ and K^+ permeability cannot be ruled out. An inhibitor of the K^+ channel, quinine, blocked the biphasic hyperpolarizations (Fig. 4*C*). Another K^+ channel blocker, Ba^{2+}, was also effective in inhibiting the hyperpolarizations (data not shown). An inhibitor of the Cl^- channel, SITS, abolished selectively the interposing depolarizing phase, thereby exhibiting a prolonged monophasic hyperpolarizing response (Fig. 4*D*). In contrast, the electrical response was little affected by frusemide, an inhibitor of the electroneutral Cl^- transport and Ca^{2+}-activated Cl^- conductance (0·1–1 mM: data not shown). These results suggest that a hypotonic challenge gives rise to a prolonged increase in the quinine-sensitive K^+ conductance followed by a subsequent, transient increase in the SITS-sensitive Cl^- conductance.

Activation of K^+ and Cl^- currents associated with the regulatory volume decrease

The voltage clamp studies were performed in large fused Intestine 407 cells during the perfusion of a hypotonic saline to distinguish between the separate currents carried by K^+ and Cl^- ions involved in the RVD. Figure 5*A* showed changes in membrane currents (lower record) when the membrane potential was clamped at −45 mV (upper record) before and after the exposure to a hypotonic solution (arrow). In order to minimize changes in the intracellular ionic concentration, the holding current was intermittently released. With this procedure, the underlying potential profile could also be monitored simultaneously (Fig. 5*A*, upper record). Following the exposure to a hypotonic solution, the resting inward current (arrowhead in Fig. 5*A*, lower record) was progressively diminished in association with the initial hyperpolarizing response, and marked inward currents occurred during the subsequent depolarizing phase. The same procedure was repeated at different holding potential levels. Figure 5*B* and *C* illustrate changes in the membrane currents (subtracting the resting current) at the maximum hyperpolarizing (first-phase) and depolarizing (second-phase) responses as a function of the membrane potential, respectively. The reversal potentials estimated from these plots were about −80 mV for the initial hyperpolarizing phase (○) and about −27 mV for the subsequent depolarizing phase (△). A tenfold increase in the K^+ concentration shifted the reversal potential of the initial phase by about 50 mV (●) without significant alteration in that of the subsequent phase (□), whereas a decrease in the Cl^- concentration by one-tenth altered exclusively the latter by about 42 mV (▲). These results indicate that K^+ and Cl^- are the major carriers of the first- and second-phase currents, respectively.

Ca^{2+}-sensitivity of volume-regulatory K^+ and Cl^- conductances

The deprivation of extracellular Ca^{2+} with 1 mM-EGTA abolished the initial hyperpolarizing response without affecting the second phase of responses, irrespective of extracellular Cl^- concentrations (Fig. 4*E* and *F*).

Under whole-cell recordings, current changes similar to those obtained by two-microelectrode voltage clamp studies were observed both in relatively small fused Intestine 407 cells and in normal cells without PEG treatment after the exposure to a hypotonic solution. When intracellular Ca^{2+} ions were moderately buffered with

1 mM-EGTA in the dialysing pipette solution, the initial outward current as well as the corresponding hyperpolarization was still induced, though the rate was slowed, after a hypotonic challenge (Fig. 6*A*). Both of them were, however, almost completely suppressed, when most intracellular Ca^{2+} ions were chelated with a high concentration of EGTA in the pipette solution (Fig. 6*B*). In contrast, the late currents associated with the depolarizing phase were preserved under these conditions (Fig. 6*A* and *B*).

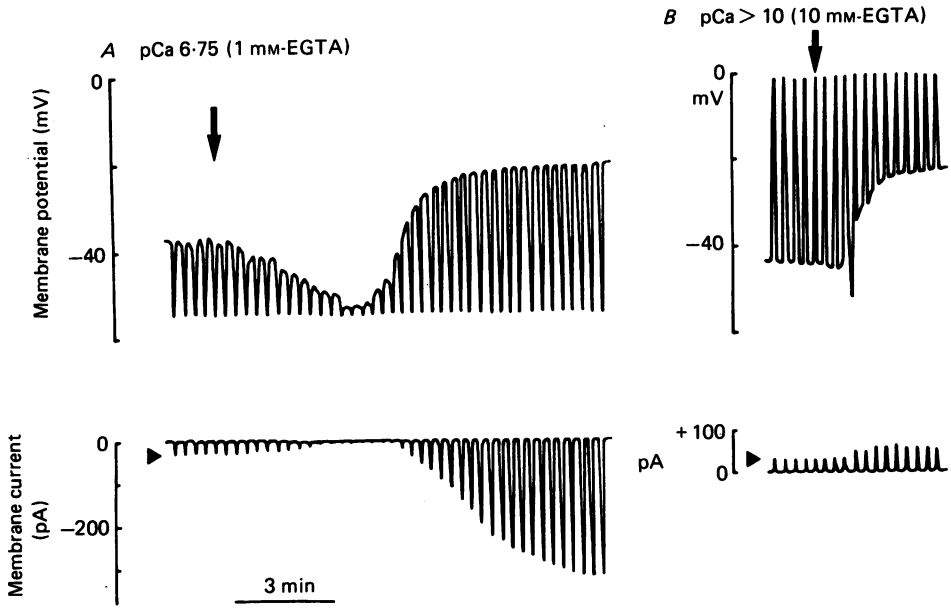

Fig. 6. Effects of Ca^{2+}-buffering capacity within the cells on changes in the membrane current in response to a hypotonic challenge under tight-seal whole-cell recordings. Membrane current recordings at certain holding potentials (−55 mV in *A*, 0 mV in *B*) and zero-current potential measurements were alternately made in relatively small fused cells containing several nuclei (cell size: *A* > *B*). The cytosolic free Ca^{2+} concentration was buffered at pCa 6·75 with 1 mM-EGTA (*A*) or pCa > 10 with 10 mM-EGTA (*B*). Under these conditions, the late hyperpolarization following the second-phase depolarization (see Fig. 4*A*) was never observed. Arrows and arrow-heads are the same as in Fig. 5*A*.

To test whether a rise of intracellular Ca^{2+} ions can activate K^+ and/or Cl^- channels, Ca^{2+} ions were injected into the cells by electrophoresis. The Ca^{2+} injection consistently evoked a monophasic hyperpolarization with a concomitant decrease in the input membrane resistance (Fig. 7*A*, at thin arrows). In contrast to the response to a hypotonic challenge (Fig. 7*A*, at a thick arrow), however, no depolarizing phase followed the Ca^{2+}-induced hyperpolarization even in a low-Cl^- solution. The Ca^{2+}-induced hyperpolarization was abolished by quinine (Fig. 7*B*) but not by SITS (Fig. 7*C*). These results show that elevation of the cytoplasmic Ca^{2+} activates the K^+ conductance but not the Cl^- conductance in Intestine 407 cells.

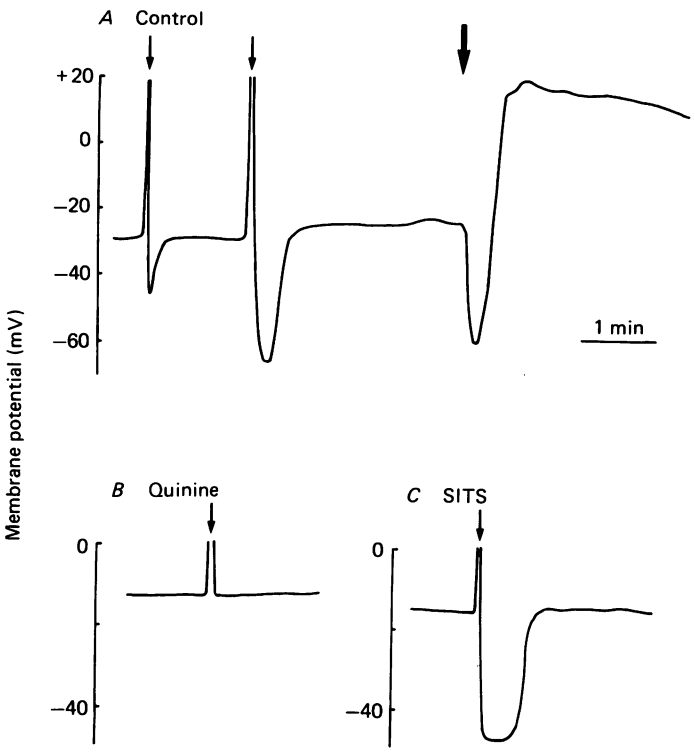

Fig. 7. Effects of intracellular Ca^{2+} injections on the membrane potential. *A*, in a giant cell incubated in a low-Cl^- (6 mM) isotonic saline solution, Ca^{2+} injections were made by applying outward currents of 5 nA (at the first thin arrow) and 10 nA (at the second thin arrow) through an additional microelectrode filled with 0·1 M-$CaCl_2$. Application of inward currents on 5–10 nA was ineffective (not shown). The same cell showed a biphasic response upon exposure to a low-Cl^- hypotonic saline solution (at a thick arrow) in contrast to the monophasic hyperpolarizing response to the Ca^{2+} injection. *B*, in a giant cell incubated in the control isotonic saline solution with quinine (0·4 mM, 12 min), a Ca^{2+} injection was made by applying 10 nA (at thin arrow). *C*, in a giant cell incubated in the control isotonic saline solution with SITS (0·1 mM, 10 min), a Ca^{2+} injection was made with 10 nA (at thin arrow). Each trace represents one of the essentially same duplicate data.

DISCUSSION

Swelling of Intestine 407 cells caused by reduction in the osmolarity of bathing solution was followed by rapid volume readjustment, or a regulatory volume decrease (Fig. 2*B*). It is known that the RVD is accomplished by a loss of KCl and water within the cell in many cell species (Kregenow, 1981; Spring & Ericson, 1982; Grinstein *et al.* 1984). Two types of mechanisms, electroconductive separate pathways (Grinstein *et al.* 1982*a*; Hoffmann *et al.* 1984; Sarkadi *et al.* 1984*a*) and electroneutral co-transport pathways (Schmidt & McManus, 1977; Cala, 1980; Dunham & Ellory, 1981; Parker, 1983; Larson & Spring, 1984; Lauf, 1985), have been proposed to account for the KCl fluxes during the RVD. The present voltage clamp studies in Intestine 407 cells provide direct evidence for activation of separate quinine-sensitive K^+ and SITS-sensitive Cl^- currents during the RVD.

In a variety of cell species, it has been suggested that Ca^{2+}-activated K^+ channels are involved in the RVD mechanism, because inhibitors of Ca^{2+}-dependent K^+ channels, such as quinine, suppressed volume regulation upon a hypotonic challenge (Hoffmann *et al.* 1984; Sarkadi, Mack & Rothstein, 1984*b*; Sarkadi *et al.* 1985; Foskett & Spring, 1985). However, pharmacological approaches may have limitations due to non-specific effects. In addition, quinine was reported to inhibit Ca^{2+}-independent (but not Ca^{2+}-activated) K^+ channels in a cell line (Findlay, Dunne, Ullrich, Wollheim & Petersen, 1985). Three pieces of evidence obtained in the present study for the involvement of Ca^{2+}-activated K^+ channels in the RVD may be summarized as follows: first, the quinine-sensitive hyperpolarizing response to a hypotonic challenge was abolished by the deprivation of extracellular Ca^{2+} ions (Fig. 4*E* and *F*). Second, cytosolic Ca^{2+} chelation inhibited the K^+ current recorded by the whole-cell patch clamp method (Fig. 6*B*). Third, intracellular Ca^{2+} injection induced a quinine-sensitive hyperpolarization (Fig. 7).

Cytosolic Ca^{2+} ions were also suggested to be involved in the activation of Cl^- conductance during the RVD based on the observation that A23187 can induce activation of the Cl^- transport as well as shrinkage of Ehrlich ascites tumour cells (Hoffmann *et al.* 1986). On the other hand, it was also reported that the volume-regulatory anionic pathway can still be activated in Ca^{2+}-depleted cells (Grinstein *et al.* 1982*b*). Our four lines of evidence indicate that the volume-regulatory Cl^- channels are independent of cytosolic Ca^{2+} ions. (a) The SITS-sensitive depolarizing phase in response to a hypotonic challenge was preserved under the Ca^{2+}-free bathing conditions which prevented the Ca^{2+}-dependent K^+ channel activation (Fig. 4*E* and *F*). (b) Cl^- channels could be activated by a hypotonic challenge in the whole-cell recordings, even when intracellular Ca^{2+} ions were chelated (Fig. 6*B*). (c) Intracellular Ca^{2+} injections failed to activate the Cl^- conductance (Fig. 7). (d) Frusemide, which is known to inhibit Ca^{2+}-activated Cl^- channels in an epithelial cell species (Evans *et al.* 1986), did not inhibit the RVD process nor the corresponding electrical membrane responses. In addition, a preliminary observation has shown that a sizeable increase in the cytosolic free Ca^{2+} concentration was associated with the initial hyperpolarization but not with the second-phase depolarization (Hazama & Okada, 1987*b*).

Since the second-phase depolarizing response due to Cl^- channel activation was usually preceded by a hyperpolarization due to K^+ channel activation, one might argue that the operation of Cl^- channel may be triggered by a voltage-gated mechanism. However, the volume-regulatory Cl^- conductance could develop at a variety of holding potential levels (-80 to $+40$ mV; Fig. 5) and even without preceding hyperpolarizations (Fig. 4*F*). Recently, it has been suggested that metabolites of arachidonic acid are involved in modulation of the Cl^- transport associated with the RVD (Lambert, Hoffmann & Christensen, 1987). Further electrophysiological investigations are required for identification of the factor(s) involved in the Cl^- channel activation during the RVD.

It is known that an increase in the cytosolic free Ca^{2+} ions takes place in some phase of the RVD process (Cala *et al.* 1986; Wong & Chase, 1986). From the dependence of the RVD on extracellular Ca^{2+}, it is conceivable that the Ca^{2+} ions may originate from the extracellular space in the case of human lymphocytes

(Sarkadi *et al.* 1984*b*) and frog urinary bladder (Wong & Chase, 1986; Davis & Finn, 1987). On the other hand, Ca^{2+} ions released from some intracellular stores could be responsible for the RVD in duck erythrocytes (Kregenow, 1971), Ehrlich ascites tumour cells (Hoffmann *et al.* 1984) and MDCK cells (Roy & Sauvé, 1987), since the RVD was independent of the extracellular Ca^{2+} in these cells. In Intestine 407 cells, the RVD process and the volume-regulatory K^+ conductance were both sensitive to extracellular Ca^{2+} ions (Figs 3*C*, 4*E* and *F*). Therefore, increased cytosolic Ca^{2+} ions responsible for the RVD would be extracellular in origin. Stretch-activated cation channels may participate in Ca^{2+} influxes upon swelling of the cells, as shown in choroid plexus epithelial cells (Christensen, 1987).

Small intestinal epithelial cells can actively take up many organic solutes in a Na^+-dependent manner and accumulate them within the cytoplasm against their electrochemical gradients. Thus, enterocytes should suffer from perturbation in the intracellular osmolarity. In fact, increases in cell-water content and volume were observed in enterocytes during active solute transport (Schultz, Fuisz & Curran, 1966; Csáky & Esposito, 1969; Armstrong, Musselman & Reitzug, 1970; Okada, 1979). Taken together, volume-regulatory ion channels appear to operate *in situ* under physiological conditions. Ba^{2+}-sensitive K^+ conductance activation was indeed observed in *Necturus* small intestinal epithelial cells after addition of galactose to the mucosal solution (Lau, Hudson & Schultz, 1984, 1986). Activation of Ca^{2+}-dependent K^+ channels was also suggested in rabbit enterocytes exposed to amino acids or sugars (Sepúlveda, Burton & Brown, 1986). Whether the volume-regulatory ion channels are located on the luminal or basolateral membrane is not clear from the present study, since the electrical recordings were made at the whole-cell level in Intestine 407 cells and the cell is rather an undifferentiated cell line lacking the polarity. In a variety of epithelia, however, volume-regulatory KCl exit pathways were suggested to reside in the basolateral membrane (Larson & Spring, 1984; Lau *et al.* 1984; Davis & Finn, 1987).

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