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Hypotonically Activated Chloride Current in HSG Cells

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Abstract. Hypotonically induced changes in whole-cell currents and in cell volume were studied in the HSG cloned cell line using the whole-cell, patch clamp and Coulter counter techniques, respectively. Exposures to 10 to 50% hypotonic solutions induced dose-dependent increases in whole-cell conductances when measured using K^+ and Cl^- containing solutions. An outward current detected at 0 mV, corresponded to a K^+ current which was transiently activated, (usually preceding activation of an inward current and had several characteristics in common with a Ca^{2+}-activated K^+ current we previously described in these cells. The hypotonically induced inward current had characteristics of a Cl^- current. This current was inhibited by NPPB (5-nitro-2-(3-phenyl-propylamino)-benzoate) and SITS (4-acetamido-4′-isothiocyanostilbene), and its reversal potentials corresponded to the Cl^- equilibrium potentials at high and low external Cl^- concentrations. The induced current inactivated at voltages greater than +80 mV, and the *I-V* curve was outwardly rectifying. The current was unaffected by addition of BAPTA or removal of GTP from the patch pipette, but was inhibited by removal of ATP or by the presence of extracellular arachidonic acid, quinacrine, nordihydroguairetic acid, and cytochalasin D. Moreover, exposure of HSG cells to hypotonic media caused them to swell and then to undergo a regulatory volume decrease (RVD) response. Neither NPPB, SITS or quinine acting alone could inhibit RVD, but NPPB and quinine together totally inhibited RVD. These properties, plus the magnitudes of the induced currents, indicate that the hypotonically induced K^+ and Cl^- currents may underlie the RVD response. Cytochalasin D also blocked the RVD response, indicating that intact cytoskeletal F-actin may be required for activation of the present currents. Hence, our results indicate that hypotonic stress activates K^+ and Cl^- conductances in these cells, and that the activation pathway for the K^+ conductance apparently involves $[Ca^{2+}]$, while the activation pathway for the Cl^- conductance does not involve $[Ca^{2+}]$ nor lipoxygenase metabolism, but does require intact cytoskeletal F-actin.

Key words: Chloride channels — Hypotonic stress — Salivary glands — Ductal cells — Patch clamp — Cytoskeleton

Introduction

Many types of cells are capable of regulating their volumes when exposed to anisotonic solutions. Regulatory volume decrease (or RVD) is the process by which cells return to their original volumes following cell swelling induced by exposure to a hypotonic solution. The RVD process is usually associated with loss of K and Cl ions from the cells. This loss can occur through a number of mechanisms including: (i) K^+/H^+ and Cl^-/HCO_3^- exchangers, (ii) a coupled K^+ and Cl^- cotransport system, or (iii) separate conductive pathways for K and Cl ions (Hoffmann & Simonsen, 1989; Lewis & Donaldson, 1990). With regard to the latter, volume-sensitive K^+ conductances have been described in several kinds of cells (Hazama & Okada, 1988; Christensen & Hoffmann, 1992; Samman et al., 1993). Similarly, volume-sensitive Cl^- conductances have been reported in several epithelial and nonepithelial cell types such as Ehrlich ascites cells (Hoffmann & Simonsen, 1989), chromaffin cells (Doroshenko & Neher, 1992), rat colonic epithelial cells (Diener, Nobles & Rummel, 1992), cultured human intestine 407 cells (Kubo & Okada, 1992), human colonic T84 cells (Worrel et al., 1989), human neutrophils (Stoddard, Steinbach & Simchowitz, 1993), human keratinocytes (Rugolo et al., 1992), mouse neuroblastoma F 11 cells (Pollard, 1993) and cultured rat epididymal cells (Chan et al., 1993). Despite a few minor differences, the volume-activated Cl^- conductances in all these cells are ac-

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tivated by swelling (or by exposure to hypotonic media), have outwardly rectifying *I-V* curves, are inhibited by common Cl^- channel blockers, and are generally independent of changes in cytoplasmic Ca^{2+} ion concentration ($[Ca^{2+}]$). The present communication describes hypotonically activated K^+ and Cl^- current and cell volume changes that occur in HSG cells, a cell line derived from an irradiated, histologically benign, human submandibular gland by Shirasuna et al. (1981). The activated K^+ current appears to flow through Ca^{2+}-activated K^+ channels, which we have previously described (Izutsu et al., 1994). The activated Cl^- current shares many of the properties previously described for hypotonically activated Cl^- currents in other cells, and our cell volume findings indicate that these currents are involved in the RVD response of HSG cells.

Cl^- current and RVD responses in HSG cells are of interest because they may elucidate the ion transport capabilities and functions of intercalated duct cells. The latter cells are normally inaccessible for physiological study because of their relatively small numbers and unique location positioned between acini and larger ducts. Hence, a cell line representative of intercalated duct cells is of considerable interest and value. Both ultrastructural and immunohistochemical findings support the premise that HSG cells are derived from, and therefore functionally representative of, intercalated duct cells (*reviewed in* Patton & Wellner, 1993). Moreover, expression of the proteins: carcinoembryonic antigen, secretory component and lactoferrin, indicates that HSG cells retain some differentiated properties normally expressed by intercalated duct cells in vivo. Thus, their ion transport and volume regulatory capabilities may elucidate the role played by intercalated duct cells in salivary gland fluid and ion secretion processes.

Materials and Methods

Cell Culture

HSG cells were very kindly provided by Dr. Mitsunobu Sato of the Second Department of Oral and Maxillofacial Surgery, Tokushima University, Japan. Cells were cultured at 37°C in 5% CO_2 in minimum essential medium with Earl's balanced salt solution (EMEM, Biofluids, Rockville, MD). The medium was supplemented with 10% fetal calf serum (FCS, GIBCO), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from GIBCO, Grand Island, NY). Cells were fed twice per week and propagated when confluent. Briefly, cells were propagated by removing the medium, washing the flask with sterile medium, then treating the flasks for 2 min with 2 ml of 0.25% trypsin/1.0 mM EDTA (GIBCO). The trypsin was then inactivated with the medium containing 10% FCS, the suspension centrifuged, and the cell pellet resuspended in medium and counted. Cells were reseeded at 1.5×10^4 cells/35 mm culture dish (Corning, Corning, NY) and cultured for 48 hr before use.

Patch Clamp Experiments

A recording chamber was applied to the culture dish by removing cells surrounding the sampling area with a paper wad, and applying a Sylgard (Corning) chamber with a 1 cm square perfusion area. Membrane currents were measured using the whole-cell voltage clamp configuration with an Axopatch 1D voltage clamp amplifier controlled with Basic-Fastlab software, an IBM-compatible computer, and an Indec IBX interface box. Outward current was denoted as positive. Whole-cell currents were monitored at −39 or −40 mV holding potential, with 100 msec pulses to 0 mV (approximately the equilibrium potential for Cl^- when using the Cl^- containing solutions) and −90 mV. Thus, currents measured at 0 mV are usually due to ions other than Cl^-, while those measured at −90 mV would include Cl^- channel currents. K^+ current was measured as the 0 mV current with K^+ present in the (internal) pipette solution. When only Cl^- currents were measured, Cs^+ and NMG^+ replaced K^+ and Na^+, respectively, in the internal and external solutions. The pulses were repeated every 3 sec (Fig. 1*A*). *I-V* curves were usually generated using a symmetrical staircase pulse pattern consisting of a rising phase of 10 voltage steps, with step changes of 10 or 20 mV, and 10 msec duration per step (Fig. 1*B*). The falling phase of the pattern reversed the rising phase, and the currents obtained at each voltage for the rising and the falling phases were averaged. The pattern was repeated every 600 msec, and the resulting current was sampled at 1 kHz. A voltage clamp protocol with a longer pulse duration was used to test for voltage-dependent channel inactivation. Twelve 100 msec pulses stepped from the holding voltage (−80 mV) to −120 mV to +120 mV in 20 mV increments were used to generate these *I-V* curves. All experiments were performed at room temperature. In all cases, background isotonic currents were subtracted from the hypotonically activated current. Junction potentials were measured using a Beckman ceramic junction, saturated KCl reference electrode, and all signals corrected accordingly.

Solutions

Cells were perfused at about 2 ml/min with a usual control medium of (in mM) 140 *N*-methyl-D-glucamine (NMG), 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose. The pH of the extracellular solution was adjusted to 7.4 by adding 140 mM of HCl, thus bringing total Cl^- ion concentration to 151 mM in the extracellular solution. The patch pipettes were usually filled with (mM) 135 CsCl, 2 Na_2ATP 0.3 H_2O, 7 HEPES (Acid), 2 $MgCl_2$, 0.1 KEGTA, and 0.2 GTP. The pH was adjusted to 7.4 with CsOH. Pipette resistance averaged about 3–5 MΩ, and series resistances were typically about 10 MΩ. We usually replaced intracellular K^+ with Cs^+ and extracellular Na^+ with *N*-methyl-D-glucamine in order to make Cl^- the predominant current carrier. (We previously described the K^+ channels present in these cells, Izutsu et al., 1994, and we have unpublished evidence for a nonspecific cation channel as well.) When experiments were performed to detect K^+ currents, KCl and KOH replaced the Cs salts, and NaCl replaced NMG^+ and gluconate. Gluconate replaced Cl^- in experiments to study the Cl^- selectivity of the hypotonically activated current. Hypotonic solutions were obtained by diluting the control perfusion medium by 10, 20, 30 or 50% with distilled water. All drugs were dissolved in the external medium unless otherwise specified. NPPB, SITS, arachidonic acid, and cytochalasin D were first made as concentrated solutions in dimethylsulfoxide (DMSO), then diluted to their final concentrations in the bathing solution. Charybdotoxin was assumed to be the active component in venom from the scorpion *Leiurus quinquestratus hebraeus* (V1755, Sigma Chemical, St. Louis, MO) and the dose was 33 μg toxin/ml.

Fura-2 Measurements

The intracellular $[Ca^{2+}]$ was determined using the calcium-sensitive fluorescent dye, Fura-2/AM, as previously described (Izutsu et al.,

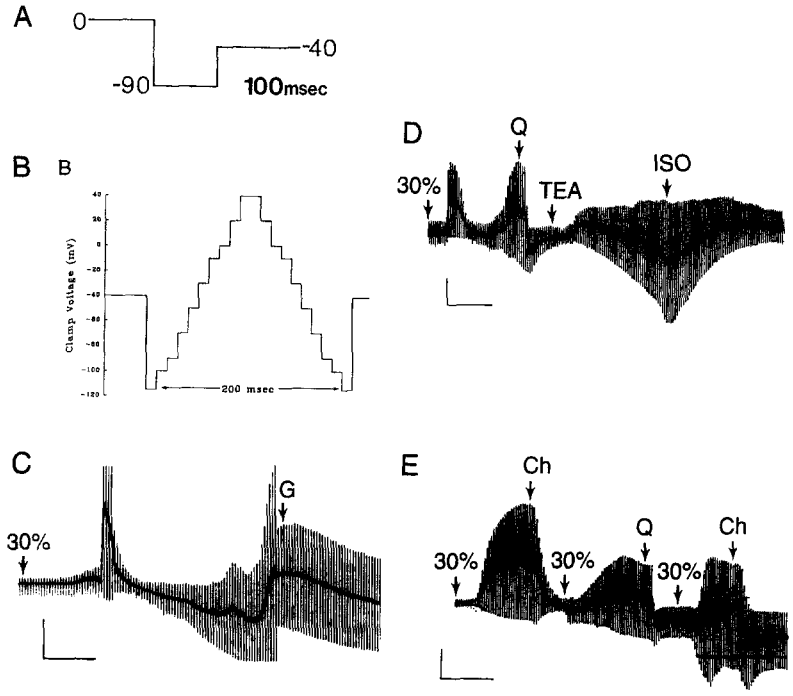

Fig. 1. Hypotonically induced whole-cell currents are sensitive to quinine and charybdotoxin but not TEA. (*A*) Whole-cell conductance was monitored by sequentially clamping the cell at 0 and −90 mV for 100 msec from a holding voltage of −40 mV. (*B*) *I-V* curves were generated using short duration voltage clamp steps and averaging the currents obtained during the increasing and decreasing steps. (*C*) Representative hypotonically induced whole-cell currents obtained in the presence of K and Cl ions using the voltage clamp pattern of *A*. Cell was exposed to 30% hypotonic solution at the arrow, and at *G* the gain was reduced by 50%. (*D*) Hypotonically induced currents as in *C*. Cell perfused with hypotonic solution containing quinine (0.5 mM) at *Q*, and tetraethylammonium ion (5 mM) at *TEA*, and reperfused with isotonic solution at *ISO*. (*E*) Hypotonically induced whole-cell currents as in *C*. Cell perfused with hypotonic solution containing charybdotoxin (scorpion venom) or quinine (0.5 mM) at *Ch* and *Q*, respectively. Calibration bars are 1 min and 200 pA for *C* and 500 pA for *D* and *E*.

1994). Briefly, confluent cells from two 100 mm plates were washed with 4 mM EGTA (pH 7.4) in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution, and then incubated in the same medium for 10 min at 37°C. Released cells were centrifuged, then resuspended in 5 ml of control perfusion medium, and 2.5 μl of Fura-2/AM (1 μM final) in DMSO added to the cell suspension. Following a 30-min incubation in the dark at room temperature, extracellular dye was removed by centrifugation and two subsequent washings with control perfusion medium. Cells were resuspended in 17 ml of control perfusion medium, and 2 ml aliquots were used in each intracellular $[Ca^{2+}]$ measurement. Intracellular $[Ca^{2+}]$ determinations were performed at room temperature, using an SLM/Aminco DMX-1000 fluorometer with a stirred cuvette. The Fura-2 ratio method (Grynkiewicz, Poenie & Tsien, 1985) was used to calculate intracellular $[Ca^{2+}]$, with fluorescence monitored at 505 nm, excitation at 340 and 380 mm and a chopper speed of 300 Hz. The *in situ* Mn^{2+}/DTPA (diethylenetriaminepentacetic acid, Sigma) technique was used to assess the contribution of extracellular dye to the Ca^{2+} signal at the beginning and end of each run. Fluorescence due to extracellular dye was estimated by quenching the signal with Mn^{2+} (43 μM), and then adding DTPA (64 μM) to chelate the Mn^{2+}. Background fluorescence from the cells in the absence of dye was subtracted. Dye fluorescence at a saturating $[Ca^{2+}]$ was determined (after the second Mn/DTPA treatment) by releasing the dye with digitonin (50 μg/ml). Dye fluorescence at nominally zero $[Ca^{2+}]$ was then determined at the end of each run by chelating the calcium with 5 mM EGTA plus Trizma base (pH 8.5 final).

Cell Volume Measurement

The volume of HSG cells was measured using a suspension of single cells. Cell suspensions were prepared from confluent HSG cell cultures by incubating with a solution consisting of 0.25% Trypsin and 1 mM EDTA in Hank's balanced salt solution (without Ca^{2+} or Mg^{2+}) for 3 min. After centrifugation, the cells were resuspended at a density of 4–8 million cells per ml in the usual NMG-containing, extracellular solution but with 5% fetal calf serum. The cells were allowed to recover in this medium for at least 1 hr at room temperature in a siliconized flask with gentle shaking.

Cell volume was determined using a Coulter Multisizer Model 0646 with sampling unit (stand II). The Accucomp 4.1 computer software was used to obtain and analyze the data. The instrument was calibrated and the calibration linearity verified using latex beads of known diameters. All measurements were made using the 100 μm aperture and the narrow distribution range. Cell volume measurements were based on the medians of distributions obtained with 20,000 to 25,000 counts. In experiments to test effects of cytochalasin D, NPPB or quinine on RVD, cells were preincubated with these agents for 3–5 min in isotonic media before transferring to hypotonic media for cell volume measurement. Cell volumes in hypotonic media were normalized against control cell volumes obtained in isotonic solution for the same experiment, and expressed as relative cell volumes.

Results

Whole Cell Current Results

Exposure of HSG cells to hypotonic solutions activated both outward and inward currents when measured using the K^+ and Cl^- containing solutions. The outward current was detected at 0 mV and thus corresponded to a K^+ current. Inward currents were detected at both −90 mV and at the holding potential of −39 or −40 mV. The outward current was generally activated transiently, and its activation usually preceded activation of the inward current (Fig. 1*C*). The relative maximum magnitudes of

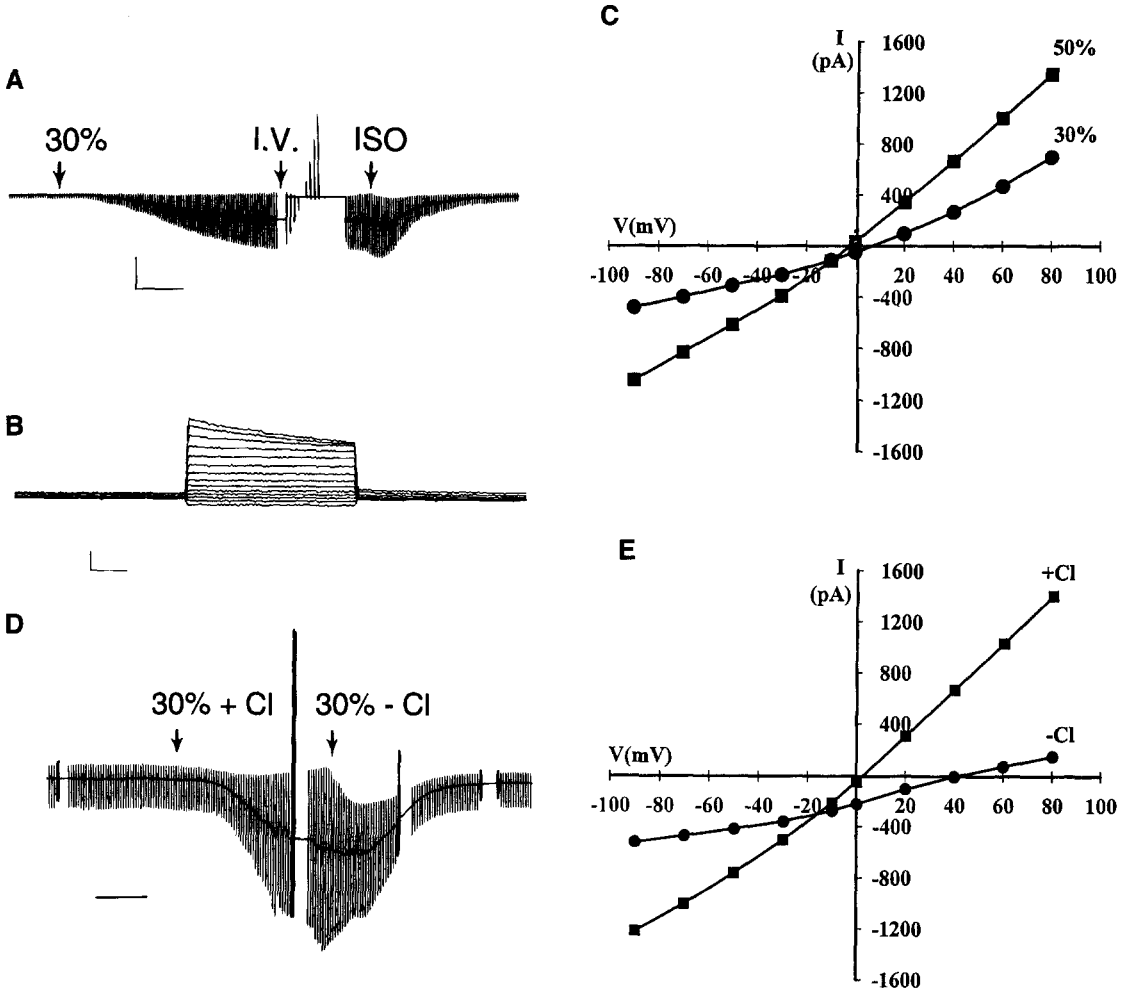

Fig. 2. Hypotonically induced inward current is Cl^- selective, and inactivates with large depolarizations. (*A*) Cell exposed to 30% hypotonic solution at 30% and reperfused with isotonic solution at *ISO* using NMGCl- and CsCl-containing solutions. Monitoring of whole-cell conductance was interrupted to obtain *I-V* curve data using long duration (100 msec) pulses. Calibration is at 50 pA and 1 min. (*B*) Whole-cell currents obtained with 100 msec voltage clamp pulses during exposure to 30% hypotonic solution. Cell voltage clamped from −120 to +120 mV in 20 mV steps (holding voltage, −80 mV). Currents obtained at membrane voltages above +80 mV inactivated with time (e.g., *see* top two current traces). Calibration bars are 20 msec and 80 pA. (*C*) *I-V* curves for the hypotonically activated current obtained with 100 msec pulses (*B*) during exposure to 30 and 50% hypotonic media. Cell capacitance was 21 pF. (*D*) Replacement of Cl^- with gluconate$^-$ in the NMG solution reduced the hypotonically activated current. First arrow indicates perfusion with 30% hypotonic NMG-Cl, and second arrow perfusion with NMG-gluconate solution. The recording was interrupted to obtain *I-V* curve data. Currents monitored as in Fig. 1*A*. Calibration bar is 1 min. (*E*) *I-V* curves for data from experiments shown in *D*. The 40 mV shift in reversal potential upon replacing extracellular Cl ions with gluconate ions was consistent with the Nernst equation.

the activated inward and outward currents varied between cells, but the two currents were generally about the same. The outward current was completely inhibited when 0.5 mM quinine (Fig. 1*D*, $n = 8$), or charybdotoxin (Fig. 1*E*, $n = 3$), but not 5 mM TEA (Fig. 1*D*, $n = 3$) was added to the perfusate. This current was also eliminated when K^+ in the pipette solution was replaced with Cs^+ (Fig. 2*A*). Since these characteristics were similar to those for a K^+ channel we previously showed was present in these cells (Izutsu et al., 1994), we then turned our attention to characterizing the inward current. Hence, the remaining experiments were performed in the absence of internal K^+ ions.

The average hypotonically induced inward current measured at −90 mV was 6.8 ± 1.0 pA/pF (mean ± SEM), and the average cell capacitance was 31.4 ± 2.6 pF ($n = 12$). The hypotonically activated inward current required 3–4 min to attain maximum activation. This increase was usually maintained for several minutes and quickly reversed by re-exposure to the isotonic medium (Fig. 2*A*). The activated inward current did tend to slowly inactivate following attainment of the maximum value (Fig. 9*C*). Moreover, this inactivation rate was much slower than that observed with Cl^- channel inhibitors (*cf.* Fig 9*C vs.* Fig 3*A*, *C*). The development of this current was accompanied by cell swelling and shrinkage that

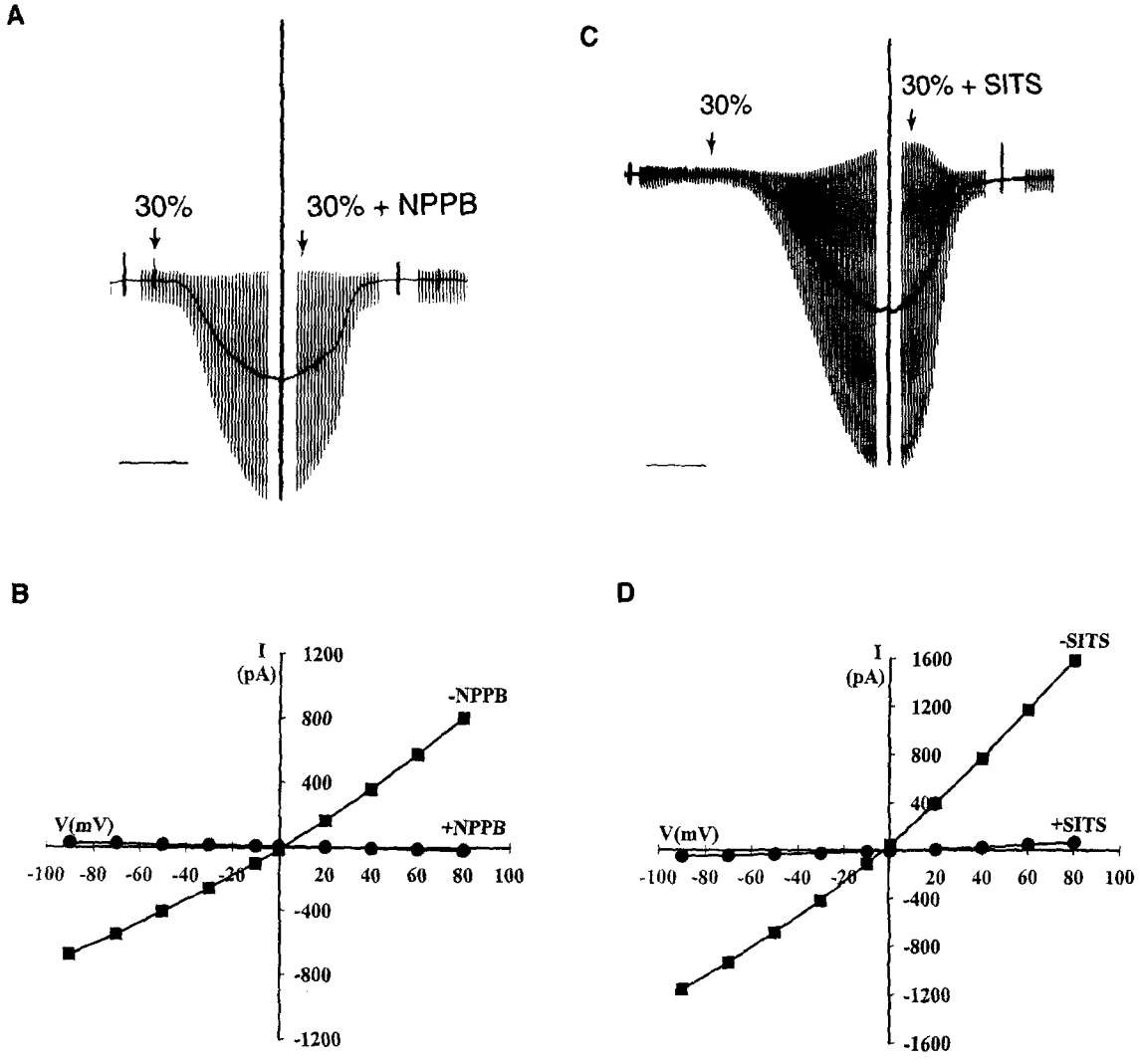

Fig. 3. The hypotonically induced inward current is sensitive to Cl^- channel blockers, NPPB and SITS. (*A*) Whole-cell current was induced with 30% hypotonic solution at first arrow, and *I-V* curve data were obtained before and after the induced response. Cell was then exposed to hypotonic solution containing 100 μM 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), and the induced response was reversed. Calibration bar is 1 min. (*B*) *I-V* curves obtained with data from experiment shown in *A*. (*C*) Hypotonically induced whole-cell current and *I-V* data were obtained in the absence and presence of 50 μM SITS as in *A*. Calibration bar is 1 min. (*D*) *I-V* curves obtained with data from experiment shown in *C*. Cell capacitance was 35 pF for *A*, 32 pF for *B*.

were measured with the Coulter Multisizer (*see below*). The *I-V* curve showed that the hypotonically activated current was outwardly rectifying, and that the current amplitude depended on the degree of hypotonicity over the range from 10 to 50% (Fig. 2*C*). The current responses, obtained with the voltage clamp pattern utilizing the 100 sec pulse duration, showed that the hypotonically induced current inactivated at membrane potentials above +80 mV (Fig. 2*B*). The activated current reversed at 3.0 ± 1.0 mV (n = 6) with the usual Cl^--containing perfusate. Replacing perfusate Cl^- with the larger gluconate ion, shifted the reversal potential to +45.0 ± 5.0 mV (n = 7) (Fig. 2*D* and *E*), consistent with the shift predicted by the Nernst equation for the Cl^- equilibrium potential. These results indicate that the hypotonically activated inward current is carried through Cl^--selective channels.

Sensitivity to Cl Channel Blockers

Exposing cells to hypotonic solutions in the presence of the Cl^- channel blocker NPPB at concentrations as low as 10 μM inhibited the hypotonically activated current. NPPB (100 μM) inhibited the activated current by 95% (Fig. 3*A* and *B*) (n = 8), and the inhibition was removed within 3–4 min of NPPB washout (*data not shown*). A similar inhibitory effect was observed with SITS, the

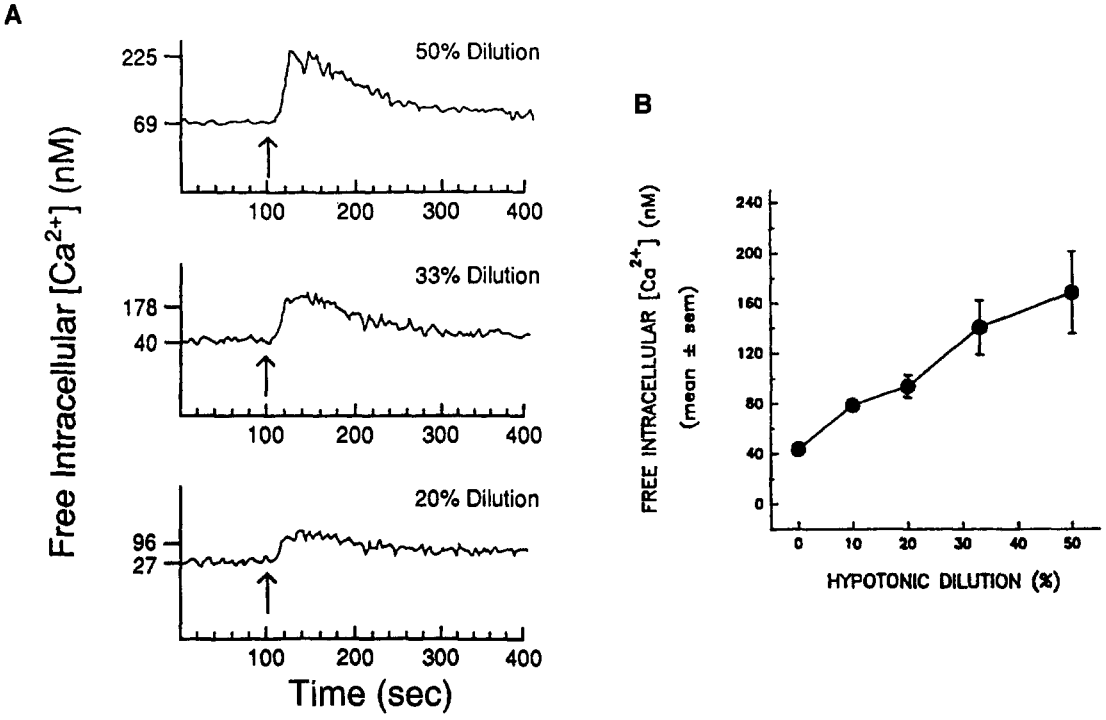

Fig. 4. (*A*) Cytoplasmic $[Ca^{2+}]$ in HSG cells is increased upon exposure to hypotonic solutions. Intracellular $[Ca^{2+}]$ measured with Fura-2 in cell suspensions as described in Materials and Methods. Exposing cells to hypotonic solutions increased cytoplasmic $[Ca^{2+}]$ in a dose-dependent fashion. (*B*) Hypotonically induced increase in cell $[Ca^{2+}]$. The curve was obtained using peak response in each hypotonic exposure measurement ($n = 4$).

stilbene-derivative Cl^- channel blocker (Fig. 3*C* and *D*). SITS (50 μM) reversibly inhibited the hypotonically activated current by 93 ± 3% ($n = 4$).

1,9-Dideoxyforskolin, a forskolin analogue which inhibits p-glycoprotein-associated Cl^- channels but not adenylate cyclase activity (Diaz et al., 1993), reversibly blocked the present hypotonically activated whole-cell currents at a concentration of 10 or 50 μM (Fig. 8*B*, $n = 4$).

Ca^{2+} Ion Independence

We were naturally curious whether the hypotonically induced inward current was dependent on an increase in cytoplasmic Ca^{2+} concentration. Exposing HSG cells to hypotonic media increased the cytoplasmic Ca^{2+} concentration in a dose-dependent manner. Figure 4*A* and *B* shows the effects of 20, 30 and 50% hypotonic solutions on intracellular Ca^{2+} concentration. Despite the induced increase in intracellular $[Ca^{2+}]$, buffering intracellular Ca^{2+} with BAPTA did not reduce or block the hypotonically activated inward current. In seven out of seven trials, cells dialyzed with 10 mM BAPTA for at least 5 min responded with activation of whole-cell Cl^- current when exposed to hypotonic media (Fig. 5). The current measured in the presence of 10 mM intracellular BAPTA had a reversal potential of +2 mV (Fig. 5*A*), and this potential was shifted to a more positive membrane voltage (+50 mV) when the extracellular Cl^--containing solution was replaced with a gluconate-containing solution (Fig. 5*B*). The current was also blocked by NPPB (Fig. 5*C* and *D*). These findings indicate that the current recorded in the presence of intracellular BAPTA was indeed a Cl^- current.

Involvement of Signaling and Cytoskeletal Components and Pathways

We found that the hypotonically induced whole cell current in HSG cells was inhibited by 30 μM arachidonic acid in the perfusion medium ($n = 5$) (Fig. 6*A*). This effect of arachidonic acid was reversible (*data not shown*), and 30 μM oleic acid, another fatty acid, was also able to inhibit the current (*data not shown*). However, quinacrine, a phospholipase A2 inhibitor, at a concentration of 10 μM blocked the hypotonically activated current by 62% as measured at −90 mV (Fig. 6*C*, $n = 3$). NDGA, a lipoxygenase blocker, completely inhibited the hypotonically induced increase in whole-cell conductance at 2.5 μM (Fig. 6*B*; $n = 4$). This effect was only partially reversible. In contrast, leukotriene D_4 had no effect on the hypotonically induced current (*data not shown*).

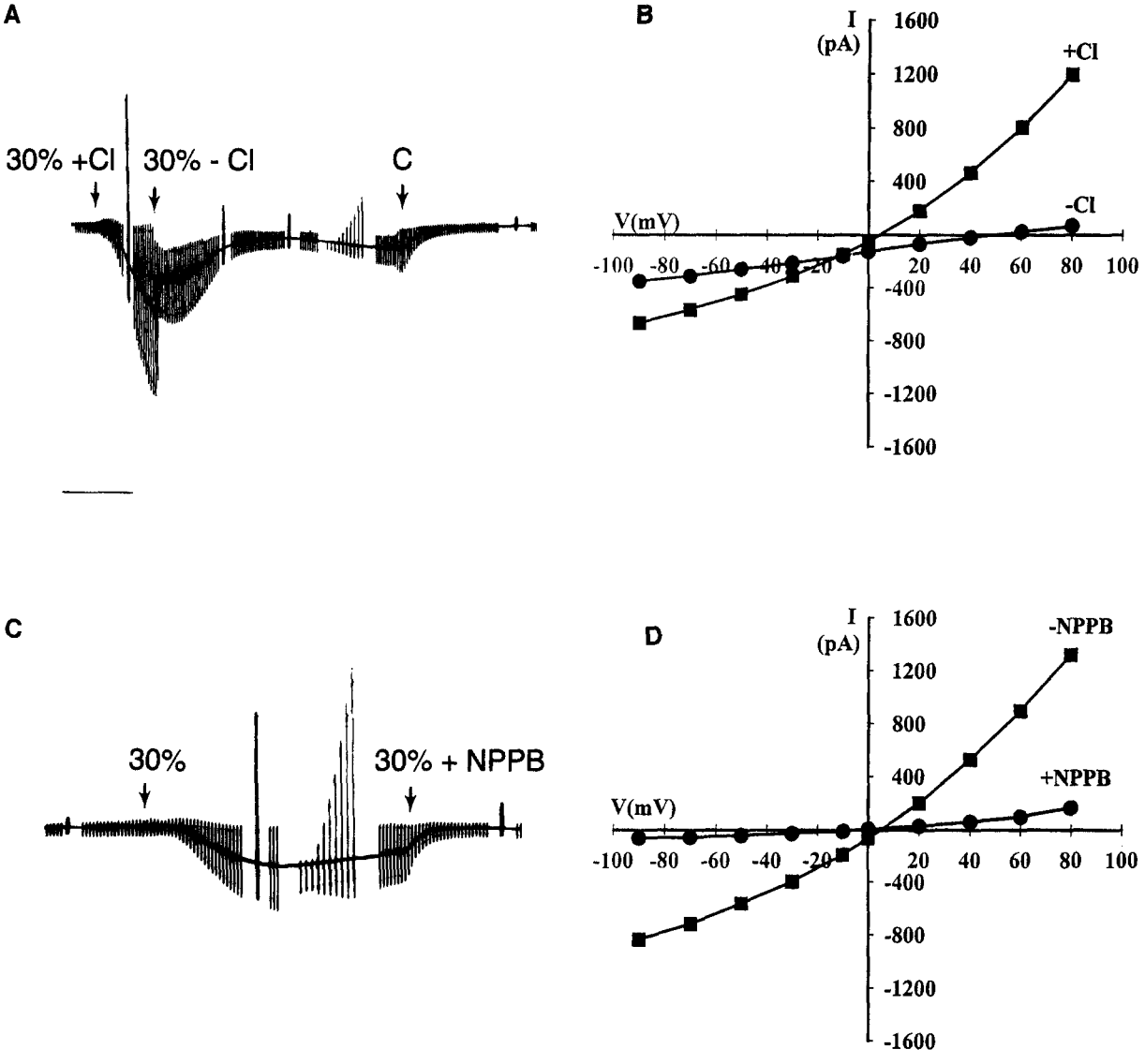

Fig. 5. The hypotonically induced inward current is not sensitive to BAPTA. (*A*) With 10 mM BAPTA in the patch pipette, the whole-cell current still increased upon exposure to a 30% hypotonic solution with Cl^- (applied at first arrow). The current decreased upon exposure to a 30% hypotonic solution without Cl^- (second arrow), and the current returned to original levels following return to control isotonic perfusate (third arrow). (*B*) *I-V* curves for experiment shown in *A*. (*C*) The induced current obtained with 30% hypotonic perfusate (applied at first arrow) was still sensitive to 100 μM NPPB (applied at second arrow). (*D*) *I-V* curves for experiment shown in *C*. Cell capacitance was 55 pF. Calibration bar is 1 min. (Records *B* and *C* were obtained from the same cell.)

Removing GTP from the intracellular solution had no effect on the hypotonically activated Cl^- current. Figure 7*A* shows the current changes induced by exposure to a 30% hypotonic solution in the absence of intracellular GTP. In these experiments, the interior of the cell was dialyzed for 5–7 min with the GTP-lacking solution before the cell was exposed to the hypotonic medium. In contrast to GTP, induction of the hypotonically activated current required ATP in the pipette solution. As noted above, the increase in Cl^- conductance could be induced repeatedly by exposing cells to hypotonic solutions with ATP in the pipette solution (Fig. 2*A*, $n = 25$). It was not possible to induce this conductance increase after dialyzing the cells for 5–7 min with no internal ATP (Fig. 7*B*).

Addition of dibutyrl-cyclic AMP (1–2 mM) to the bathing solution did not affect the currents observed in the absence of exposure to hypotonic solutions ($n = 4$, *data not shown*).

The involvement of the cytoskeleton in activating the hypotonically induced current was also investigated. Cytochalasin D which disrupts actin microfilaments was found to completely inhibit the hypotonically activated

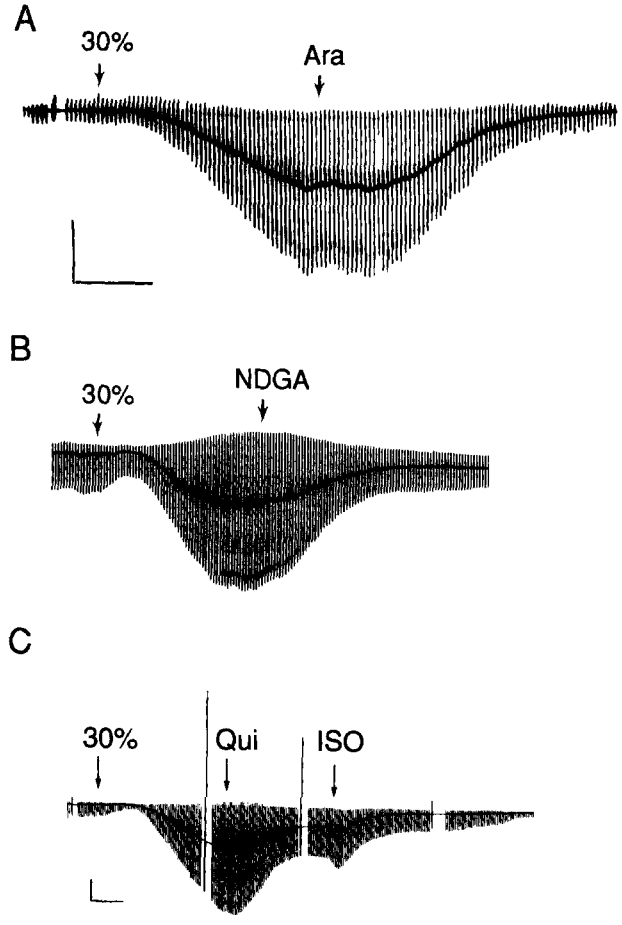

Fig. 6. The hypotonically induced inward current is sensitive to other compounds (*A*) The increase in whole-cell current induced by a 30% hypotonic solution was sensitive to 30 μM arachidonic acid applied at *Ara.* (*B*) Nordihydroguaiaretic acid (2.5 μM) applied at *NDGA*, also inhibited the induced current. (*C*) Quinacrine, 10 μM, applied at *Qui*, partially inhibited the induced current. Full reversal of hypotonic response was obtained after re-exposure to isotonic solution at *ISO*. In all cases, inhibitor were added after development of the hypotonically induced current response (perfusion initiated at 30% arrow). Calibration bars are all 1 min and 500 pA. Cell capacitance was 39 pF for *A*, 45 pF for *B*, and 26 pF for *C*.

current within a few seconds at a concentration of 1 μM (Fig. 8*A*, $n = 3$). This effect of cytochalasin D was only poorly reversible.

Cell Volume Findings

Measurements made using the Coulter counter showed that HSG cells are sensitive to osmolarity change in the suspending medium. The average cell volume in the control isotonic solution was 1,012 ± 25 fl ($n = 43$). Exposing cells to a 30% hypotonic solution resulted in rapid, transient cell swelling with most cells attaining maximum volumes within the 1 min (approximate) required to make the first volume measurement following exposure to the hypotonic solution (Fig. 9*A*). Cell volumes then slowly declined towards control values over the next 7 min. The latter process consisting of the slow decrease in cell volume constitutes the RVD response. The maximum relative volume increases observed were about half of what was expected from the osmolarity changes, but the significance of this difference could not be evaluated because of the relatively long time required to make the volume measurement with our apparatus.

Most tested Cl^- channel inhibitors did not affect the observed RVD process. NPPB, which inhibits Cl^- channels in several tissues including the present cells, had no effect on the present RVD response (Fig. 9*B*). SITS, another Cl^- channel inhibitor, also had little effect (*data not shown*). Quinine, which inhibits Ca^{2+}-activated K^+ channels in HSG cells (Izutsu et al., 1994), decreased the RVD process slightly (Fig. 9*B*). However, the combination of quinine plus NPPB nearly completely inhibited the RVD response (Fig. 9*B*). The latter finding indicates that both K^+ and Cl^- channels are activated by exposure to hypotonic medium.

Cytochalasin D also appeared to totally inhibit the RVD process, and the relative cell volume actually increased for several minutes following exposure to the hypotonic medium in its presence (Fig. 9*A*).

These findings indicate that HSG cells are sensitive to osmolarity changes in the suspending medium, and that they are capable of RVD following osmotically induced cell swelling. The RVD compensatory changes require 10 min or more to reach completion in these cells, and the process is inhibited by cytochalasin D and the combination of quinine and NPPB.

Discussion

Hypotonic Stress Induces A K^+ and A Cl-Current

Exposure of HSG cells to hypotonic solutions activated both a Cl^- and a K^+ conductance when tests were made with both ions present. The present K^+ current was similar to the muscarinically activated K^+ current described earlier (Izutsu et al., 1994) in that it was sensitive to quinine and charybdotoxin but not to TEA (Fig. 1*D* and *E*). Moreover, the previously described K^+current was activated by an increase in cytosolic [Ca^{2+}], and activation of the present K^+ current was also correlated with an increase in cytosolic [Ca^{2+}]. Also, the time course of K^+ current activation (Fig. 1*C*) was very similar to the time course of increase in cytosolic [Ca^{2+}] (Fig. 4*A*). All of these results indicate that the present hypotonically induced outward current is carried through the Ca^{2+}-activated K^+ channels that we previously described in these cells (Izutsu et al., 1994).

Several lines of evidence support the conclusion that the present hypotonically activated inward current is due

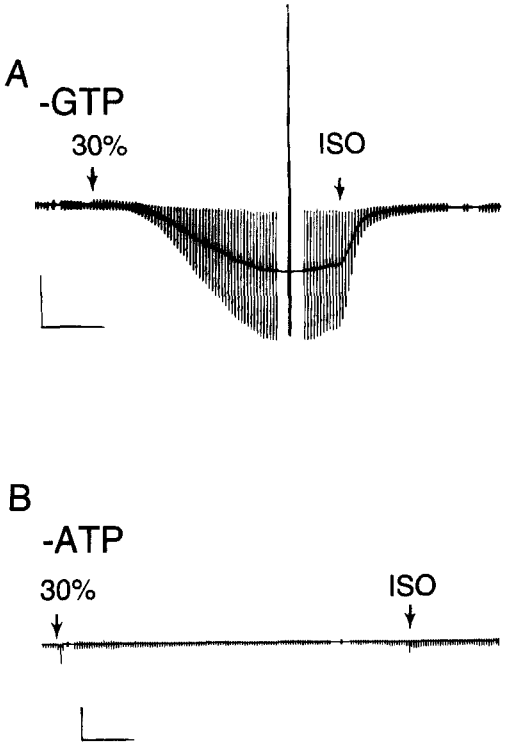

Fig. 7. GTP and ATP sensitivity of the hypotonically induced inward current. (*A*) The whole-cell current still increased upon exposure to a 30% hypotonic solution (first arrow) when GTP was not included in the pipette solution. The effect reversed upon reperfusion with control isotonic solution (*ISO*, second arrow). (*B*) In contrast, exposure to the 30% hypotonic solution was without effect (first arrow) when ATP was not included in the pipette solution. Calibration bars are 1 min and 500 pA. Cell capacitance was 26 pF for *A* and *B*.

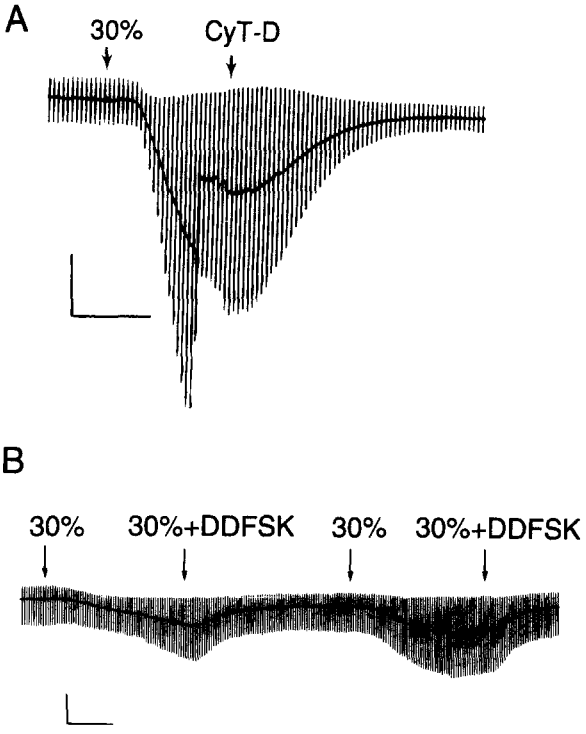

Fig. 8. Effects of cytochalasin D and dideoxyforskolin on the hypotonically induced inward current. (*A*) The increase in whole-cell current elicited by exposure to a 30% hypotonic solution (30% arrow) was inhibited by 1 μM cytochalasin D (*CYT-D* arrow). (*B*) Dideoxyforskolin (50 μM) (indicated as 30% + DDFSK) inhibited the increase in whole-cell current obtained with 30% hypotonic solution (first arrow). Washing with 30% hypotonic solution (third arrow) partially reversed the inhibition, which could be demonstrated again (fourth arrow). Calibration bars are 1 min and 500 pA for *A* and *B*. Cell capacitance was 80 pF for *A* and 37 pF for *B*.

to an increase in Cl^- conductance: (i) The reversal potential for the activated current obtained with Cl^--containing solutions corresponded to the Cl^- equilibrium potential as calculated from the Nernst equation (Fig. 2*C*). (ii) The reversal potential for the activated current was shifted towards positive membrane voltages when most of the Cl^- in the extracellular solution was replaced with the larger gluconate ion, in keeping with the shift predicted by the Nernst equation (Fig. 2*D* and *E*). (iii) The activated current was blocked by micromolar levels of NPPB and SITS (Fig. 3*A* and *C*), two classical blockers of Cl^- channels in various other tissues (Dreinhofer et al., 1988; Doroshenko & Neher 1992; Kubo & Okada 1992).

The present hypotonically induced Cl^- current also inactivated at high positive membrane potentials (i.e., at V_m greater than +80 mV; Fig. 2*B*). Such an inactivation has been reported in several other tissues (Worrel et al., 1989; Kubo & Okada, 1992; Rugolo et al., 1992). Lewis and Donaldson (1990) suggested that this property acts to limit hypotonically induced membrane depolarization. However, the physiological relevance of this inactivation in HSG cells is unknown since the Cl^- equilibrium potential is much less than the +80 mV required to induce Cl^- current inactivation (Fig. 2*B*).

Mechanism of Activation of Cl Current

The mechanism underlying activation of the hypotonically induced inward current is still not known. We investigated several possible signaling pathways for activation of this current, including an increase in Ca^{2+} ion concentration, an increase in cyclic AMP level, an increase in phospholipase A2 activity, and an involvement of the cytoskeleton.

Cytoplasmic [Ca^{2+}] has been reported to increase during the RVD process in a number of cell types (Hoffman & Simonsen, 1989; Lewis & Donaldson, 1990). This increase may be due to release of Ca^{2+} from intracellular storage sites (Cornet, Lambert & Hoffmann, 1993) and/or to Ca^{2+} entry through nonselective or other cationic channels (Christensen & Hoffmann, 1992). Similarly, the cytoplasmic [Ca^{2+}] in HSG cells increased in a dose-dependent manner when exposed to hypotonic solutions (Fig. 4). As noted earlier, the close correlation

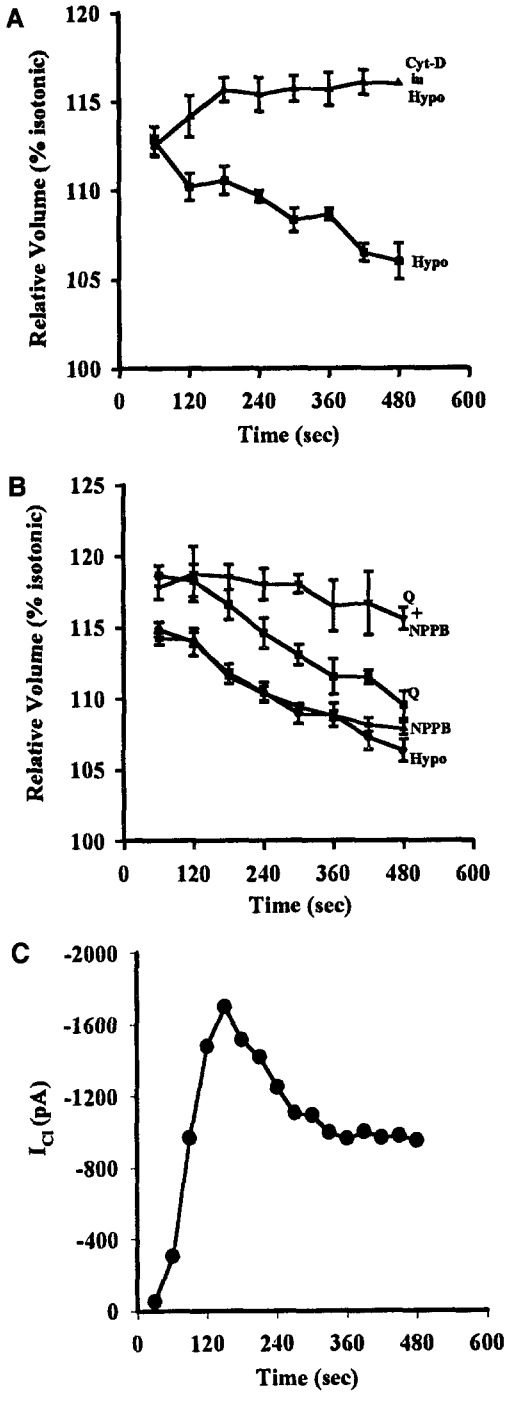

Fig. 9. (*A*) Cytochalasin D (1 μM) inhibits the hypotonically induced RVD response of HSG cells. Cell volumes increased rapidly, then decreased toward original values following exposure to 30% hypotonic solution (*Hypo* curve). The volume decrease represents the RVD process. Cytochalasin D inhibited the RVD response (*Cyt-D* curve). (*B*) NPPB (100 μM) and quinine (0.5 mM) together inhibited RVD. Quinine (*Q*) and/or NPPB were added to the cell suspension media 3–5 min before exposing cells to hypotonic media containing NPPB and/or quinine. Cells exposed to NPPB or quinine alone displayed RVD responses similar to (NPPB curve) or slightly different from (*Q* curve) the controls (*Hypo* curve). In contrast, cells incubated with both quinine and NPPB showed little RVD (*Q + NPPB* curve). (*C*) A representative time course for activation and inactivation of inward current at −90 mV during exposure to a 30% hypotonic perfusate. Cell capacitance was 55 pF.

between the temporal increases in cytoplasmic $[Ca^{2+}]$ and outward current indicates that the K^+ channel is likely Ca^{2+} activated. However, the present hypotonically induced inward current was not Ca^{2+} activated since it was inducible even when the cell interior was dialyzed with 5 or 10 mM BAPTA (Fig. 5). We previously demonstrated that this maneuver is capable of inhibiting activation of the Ca^{2+}-activated K^+ channels in these cells (Izutsu et al., 1994). Moreover, the characteristics of the current obtained with BAPTA dialysis were unchanged from those of the current obtained with 0.1 mM EGTA in the pipette solution in that the reversal voltage of the current still followed the Nernst equation for Cl^- and the current was blocked by NPPB (Fig. 5*B*). Hence, the hypotonically induced increase in Cl^- conductance in HSG cells is not Ca^{2+} activated. This finding is similar to that previously reported for a number of other cell types including human intestinal 407 cells (Hazama & Okada, 1988; Kubo & Okada, 1992), bovine chromaffin cells (Doroshenko & Neher, 1992), T-lymphocytes (Lewis, Ross & Cahalan, 1993), human neutrophils (Stoddard et al., 1993) and *Xenopus* oocytes (Ackerman et al., 1994). The present finding is not consistent with findings in fibroblasts (Mastrocola et al., 1993) and in other glandular cells (Foskett & Spring, 1985; Moran & Turner, 1993; Samman et al., 1993).

The present findings that the hypotonically induced Cl^- conductance increase is inhibited by arachidonic acid, quinacrine and NDGA are consistent with findings from several other cell types (Doroshenko & Neher, 1992; Kubo & Okada, 1992). These findings, plus the observation that indomethacin, a cyclooxygenase inhibitor, has no such inhibitory effect (Diener et al., 1992), and the observation in several tissues that LTD_4 activates RVD (Lambert, 1987) or the volume-activated Cl^- current (Doroshenko & Neher, 1992), have been interpreted as indicating that arachidonic acid can directly inhibit the Cl^- channels (Kubo & Okada, 1992), but that activation of the lipoxygenase pathway and formation of LTD_4 are responsible for activation of the hypotonically induced Cl^- current (Doroshenko & Neher, 1992; Hyun & Binder, 1993). In the present HSG cells, LTD_4 was not observed to activate this current. Thus, there is as yet no evidence that the lipoxygenase pathway is involved in activating the hypotonically induced current response in HSG cells.

Our results clearly show that ATP is required to elicit the hypotonically induced increase in Cl^- conductance in HSG cells, because the response was eliminated when ATP was removed from the pipette solution (Fig. 7*B*). In contrast, removal of GTP from the pipette solution was without effect (Fig. 7*A*). A similar ATP dependence of a hypotonically activated Cl^- conductance was also reported for T-lymphocytes (Lewis et al., 1993), but it is not yet known whether the ATP is required for phosphorylation or allosteric activation, or as an energy source for ion pumping or reorganization of cytoskeletal filaments.

Cytoskeletal Involvement in the Activation of the Cl^- Conductance and RVD

Our finding that the hypotonically induced increase in Cl^- conductance was inhibited by cytochalasin D (Fig. 8*A*) suggests that changes in the cell cytoskeleton are involved in activating the Cl^- conductance. In this regard, cytochalasin D is known to disrupt the cytoskeletal F-actin network (Shliwa, 1982), and Suzuki et al. (1993) recently showed that Cl^- channel activity in apical membranes of rabbit renal proximal convoluted tubule cells was affected by alterations of the F-actin network. Others have demonstrated that disruption of actin microfilaments inhibits RVD in gall bladder epithelial cells (Foskett & Spring, 1983), and Ehrlich ascites tumor cells (Cornet et al., 1993). Moreover, Cantiello et al. (1993) showed that human melanoma cells lacking actin-binding protein were unable to volume-regulate or to activate K^+ channels (which effect the RVD in these cells) when exposed to hypotonic solutions. Genetic rescue with actin-binding protein resulted in recovery of both responses, further indicating that the actin cytoskeleton is functionally involved in osmotically sensitive ion transport.

Our results are also consistent with the above proposed interaction between the cytoskeleton and the Cl^- channels and RVD, because cytochalasin D blocked *both* the hypotonically activated Cl^- current *and* the associated RVD response (Fig. 9*A*). Lewis and Donaldson (1990) suggested that the microfilaments may act by (i) inserting ion channels from a cytoplasmic store into the surface membrane, (ii) acting as a mechanical linkage transmitting membrane stretch directly to the channel, and activating the channel through conformational changes, or (iii) activating a second messenger system that activates or modulates the channel. Our patch clamp and cell volume findings are inconsistent with the second possibility because cell swelling (and the resulting membrane stress) were not temporally correlated with the increase in whole-cell Cl^- current. Specifically, the hypotonically activated inward current typically increased slowly over several minutes before reaching a maximum after 3–5 min and then slowly inactivating (Fig. 9*C*). In contrast, cell swelling occurred almost immediately, with cell volume and membrane stress attaining maximum values in about a minute, before decreasing over the next several minutes (Fig. 9*A*). Thus, cell swelling (and membrane stretch) were decreasing while the whole-cell current was increasing. Hence, membrane stretch cannot be a direct activator of the RVD-associated whole-cell inward current.

Involvement of K^+ and Cl^- Channels in RVD Response

The present Coulter counter results show that HSG cells are sensitive to medium tonicity changes because their cell volumes increase when they are exposed to hypotonic solutions, and that they are capable of a RVD process. The RVD process is fairly slow in these cells and still incomplete after 10 min (Fig. 9*A,B*).

The present Coulter counter and whole-cell patch clamp findings are generally consistent with one another, and with the hypothesis that K^+ and Cl^- currents effect the RVD process in these cells. Thus, the whole-cell findings indicate that exposure to hypotonic solutions activates a quinine- and charybdotoxin-sensitive K^+ current and an NPPB- and SITS-sensitive Cl^- current. Activation of both currents results in loss of cytosolic KCl and cell shrinkage. Consistent with this proposal are the Coulter counter-based findings that RVD was not inhibited by NPPB (Fig. 9*B*) nor completely by quinine (Fig. 9*B*) separately, but was completely inhibited in the presence of quinine and NPPB together (Fig. 9*B*).

Moreover, the observed K^+ and Cl^- currents are of the right magnitude to account for the RVD response. For example, in a representative experiment, a 60 pA Cl^- current was estimated to flow at a membrane potential of −60 mV in a 1,000 fl cell, after 30 sec exposure to a 30% hypotonic solution (Fig. 2*C*). This corresponds to a cell content change of about 0.6 mmol/liter/sec, or a 36 mM change in 60 sec. Thus, the observed Cl^- current alone could account for a cell volume change of about 10% over a minute. Since a similar K^+ current would also be induced, the total osmotic change could be as high as twice the latter value. Either of these rates is consistent with the magnitude of RVD changes observed in the present experiments, so the observed K^+ and Cl^- currents may be responsible for the RVD changes observed in HSG cells.

Comparison with RVD Responses in Other Cells

RVD responses are known to occur at different rates in different types of cells. The RVD response in fibroblasts is relatively fast, and is completed in 3–5 min (Rugolo, et al., 1992). This is faster than the response rate in the present HSG cells (Fig. 9*A* and *B*). In contrast, the RVD response in keratinocytes is extremely slow, with little or no RVD occurring over 30 min (Rugolo et al., 1992). Rugolo et al. (1992) attributed the difference in RVD rates to the fact that both K^+ and Cl^- channels were activated in fibroblasts, but only Cl^- channels were activated in keratinocytes. Thus, they proposed that both K^+ and Cl^- channels must be activated if appreciable RVD is to occur. The present findings are consistent with this proposal since HSG cells effect a relatively rapid RVD response (Fig. 9*A* and *B*), and both K^+ and Cl^- channels are activated.

Because HSG cells are proposed to have characteristics of salivary gland ductal cells, they would be expected to be relatively impermeable to water (Young & van Lennep, 1979). Hence, they are expected to be relatively insensitive to osmotic gradients, which would

eliminate the need for volume regulatory capabilities. The present results and those obtained in HSY cells [a salivary gland ductal cell line prepared from human parotid glands (Moran & Turner, 1993)] show that these expectations are incorrect, and that both types of cells swell and are capable of an RVD response when exposed to hypotonic solutions. Hence, both cell lines have appreciable water permeabilities. Moreover, while no experiments measuring hypotonically induced RVD have yet been performed on salivary gland acinar cells, RVD responses similar to the present ones occur in acinar clear cells from monkey eccrine sweat glands (Samman et al., 1993; Sato, Ohtsuyama & Sato, 1993), and following muscarinic stimulation of salivary gland acinar cells (Foskett, 1990; Foskett & Melvin, 1990).

Nature of the Cl^- Channel

The nature of the channels carrying the present hypotonically activated Cl^- current is still unknown. Ishikawa and Cook (1994) and Ishikawa, Cook and Young (1991) previously described depolarization-activated, outwardly rectifying Cl^- channels in HSG cells with single channel conductances of 25–75 pS. These channels share two characteristics with the present Cl^- currents: both are insensitive to cytoplasmic Ca^{2+} concentrations, and both are blocked by NPPB. However, the present Cl^- current has a more linear *I-V* relationship than that for the latter channels. Thus, these channels are likely not responsible for the present hypotonically activated Cl^- current.

Several other possibilities have been discussed for the identity of the Cl^- channel underlying the RVD-related Cl^- current. These include p-glycoprotein (Valverde et al., 1992), CIC-2 and pI_{Cln} (Paulmichi et al., 1992; Krapivinsky et al., 1994). Of these, CIC-2 has different properties from the hypotonically induced current, and pI_{Cln} is now thought not to be a channel (*reviewed in* Krapivinsky et al., 1994). In contrast, the present hypotonically activated Cl^- current shares several characteristics with the p-glycoprotein Cl^- currents in NIH 3T3 fibroblasts (Valverde et al., 1992) and HeLa cells (Diaz et al., 1993). The similarities include (i) an ATP dependence, (ii) inhibition by dideoxyforskolin, (iii) activation by hypotonicity, (iv) outwardly rectifying *I-V* curves, and (v) inactivation at high depolarizing voltages. These similarities suggest that the present hypotonically activated currents are associated with the p-glycoprotein, but further work is needed to test this relationship, especially since several investigators have concluded that the p-glycoprotein is not responsible for the RVD-associated Cl^- current in other cells (Ackerman et al., 1994; Altenberg et al., 1994).

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