slices are prepared in the tangential or coronal plane. In addition, their regular spatial arrangement, repeatability at specific locations, and occurrence in virtually every slice strongly suggest that they are not artifacts. Furthermore, optical recordings were done directly on a glass cover slip; no netting or other support with a regular geometry was used at any time. Finally, results on the specific pharmacology of domains do not support a slice artifact; whereas TTX, a Na^+ channel blocker, does not block domains, octanol and halothane, which are gap junction blockers, reversibly prevent the occurrence of domains.

25. D. O. Hebb, *The Organization of Behavior* (Wiley, New York, 1949), p. 62; G. S. Stent, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 997 (1973).

26. We thank D. Nelson for programming the simulations; E. Callaway, D. Purves, and T. Wiesel for comments on the manuscript; P. Peirce for photography; and C. Gutman for technical assistance. Supported by a Rockefeller University graduate fellowship (R.Y.), by NIH grants MH15177 (A.P.) and EY07960, and by an L. P. Markey Charitable Trust Scholar's Award (L.C.K.).

8 November 1991; accepted 3 June 1992

Chloride-Dependent Cation Conductance Activated During Cellular Shrinkage

Hsiao Chang Chan and Deborah J. Nelson*

A chloride (Cl^-)-dependent, nonselective cation conductance was activated during cellular shrinkage and inhibited during cellular swelling or by extracellular gadolinium. The shrinking-induced, nonselective cation conductance and the swelling-induced anion conductance appear to function in the regulation of cell volume in airway epithelia. The shrinking-induced cation conductance had an unusual dependence on Cl^-: partial replacement of extracellular Cl^- with aspartate reduced the magnitude of the shrinking-enhanced current without accompanying changes in the reversal potential. The Cl^- dependence of the nonselective cation conductance could provide a mechanism that tightly regulates Cl^- secretion and sodium reabsorption in cells under osmotic stress.

Exposure of cells to anisosmotic (hypotonic or hypertonic) media drives water into or out of the cell, resulting in acute swelling or shrinking (*1*). Many cells respond to these volume changes by modulating membrane conductance pathways as well as metabolic pathways that alter the concentration of intracellular solutes, thereby restoring cell volume to its original value. The cellular regulatory processes elicited after osmotic swelling or shrinkage are termed "regulatory volume decrease" (RVD) and "regulatory volume increase" (RVI), respectively. Regulation of cell volume is critical in certain pathological states such as ischemia and disturbances in cellular metabolism that are accompanied by swelling of cells. Even under physiological conditions, cells in the renal medulla and the intestine are subjected to osmotic stress induced by increased osmolarity in the extracellular fluid and the circulating blood plasma. Most actively transporting epithelial cells also experience osmotic variations as a result of the accumulation of osmotically active solutes within the cells and therefore must adapt to survive in environments both higher and lower in osmolarity than that of plasma.

Ion channels have been reported to participate in cell volume regulation by selectively governing the movement of ions into and out of cells. Both K^+ and Cl^- channels can be activated after a hypo-osmotic challenge, thereby initiating RVD. The increase in K^+ or Cl^- conductances, or both, results in the loss of KCl from the cell and a parallel reduction in cell volume. The extent of the reduction in cell volume is limited by the maintenance of a cellular membrane potential that favors the efflux of both ions (*2*, *3*). The activation of ion channels in response to a hyperosmotic challenge has not been demonstrated, although the participation of Na^+ or nonselective cation channels in RVI has been proposed (*4*). This study demonstrates the activation of a Cl^--dependent, nonselective cation conductance during cellular shrinkage. Cultured, dissociated human airway epithelial cells were studied with the whole-cell patch-clamp technique (*5*).

Activation of a current with a relatively linear current-voltage (*I-V*) relation occurred after exposure of a cell to a hypertonic bathing solution (*6*). The magnitude of the current increased as the osmotic pressure difference across the cell membrane was increased by the addition of sucrose to the external solution, suggesting that the current was associated with cellular shrinkage (Fig. 1). Current activation was reversed by decreasing the osmolarity of the bathing solution so that it was hypo-osmotic to the intracellular (pipette) solution. Isotonicity between cell interior and exterior was obtained when the bathing solution was made hypotonic by 30 mOsM to the intracellular solution, as determined by the absence of current activation under these conditions. Representative whole-cell currents after cellular shrinkage in response to step changes in membrane potential between −110 and +100 mV can be seen in Fig. 2, A and B. In these experiments, Na^+ and Cl^- were the major permeant ionic species, and current separation was made on the basis of shifts in reversal potential (*7*). The equilibrium potentials for a perfectly cation- or anion-selective conductance were +31 and −31 mV, respectively. Shrinking-induced current activation was associated with a depolarizing shift in current reversal potential (+26 ± 1 mV, n = 14) close to the predicted equilibrium potential for Na^+, indicating that the current was predominantly cation-selective.

The cation to anion selectivity was determined for the shrinking-activated conductance from current reversal potentials obtained when NaCl in the intracellular solution was substituted in various amounts with

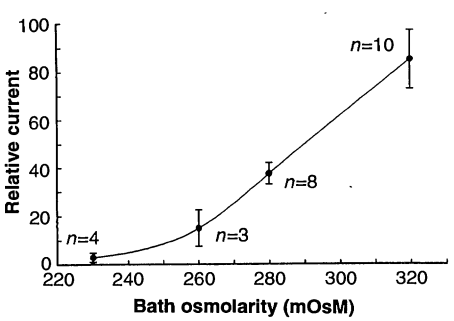

Fig. 1. Relation between whole-cell current (at 50 mV) and bath osmolarity. Experiments were performed on cultured, dissociated human airway epithelial cells with the use of the whole-cell patch-clamp technique. The pipette solution contained 40 mM NaCl, 100 mM NMDG-aspartate, 10 mM Hepes, 5 mM EGTA, 0.5 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.2. The bath solution contained 140 mM NaCl, 10 mM Hepes, 2 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.2. Extracellular solutions were made hypertonic by the addition of sucrose (standard bathing solution osmolarity was 280 mOsM). The relative current magnitude was determined as the ratio of the current for a test solution osmolarity to the average current value recorded in cells exposed to a bathing solution of 230 mOsM ($I_{\text{test osmolarity}}/I_{230\ \text{mOsM}}$). The mean current amplitude in cells exposed to the 230-mOsM bathing solution was 61 ± 7 pA at +50 mV (n = 4). Current magnitudes recorded under hypotonic conditions (bath solution <280 mOsM) were obtained after the cells had been exposed to hypertonic bathing solutions (solutions ≥280 mOsM). Inactivation of the shrinking-induced currents occurred at about 250 mOsM, as deduced from extrapolation of the curve (which corresponds to a difference of 30 mOsM between pipette and bath solutions).

Department of Medicine and Department of Neurology, University of Chicago, Chicago, IL 60637.

*To whom correspondence should be addressed.

N-methyl-D-glutamine (NMDG)–Cl^-. The cation to anion permeability ratio (P_{cation}/P_{anion}) was calculated to be 12.5. The permeability of the shrinking-activated channels to several small inorganic monovalent cations was determined in experiments in which Na^+ in the bathing solution was replaced with chloride salts of the cations to be tested. The ionic selectivity of the shrinking-induced current was calculated from shifts in the reversal potential (Fig. 2C) and determined in six cells. The relative permeability sequence (P_{cation}/P_{Na^+}) of the shrinking-induced conductance was K^+ (1.09) ≥ Na^+ (1.0) ≥ Cs^+ (0.93) > Li^+ (0.53), indicating poor selectivity among these monovalent cations, with the exception of Li^+.

Cells responded to hypotonic and hypertonic challenge with the reciprocal activation or inactivation of at least two ionic conductances. Cellular swelling induced by the reduction of extracellular NaCl to 90 mM (reduction of solution osmolarity by 100 mOsM) not only inhibited shrinking-induced current activation but also activated an outwardly rectifying anion-selective current with an accompanying shift in current reversal potential to −18 ± 4 mV (n = 7), close to the predicted Cl^- equilibrium potential of −20 mV (Fig. 3). The two volume-regulated conductances were also distinguished on the basis of their pharmacological sensitivity. The Cl^- channel blocker 4,4′-di-isothiocyanostilbene-2,2′-disulfonic acid (DIDS) inhibited the swelling-induced anion conductance (8). The shrinking-induced cation conductance was completely inhibited by the addition of gadolinium (10 μM, n = 6) to the external solution (Fig. 2A). Activation of the shrinking-induced current was detected in the presence of amiloride (100 μM) and was not sensitive to either bumetanide (100 μM, n = 4) or furosemide (100 μM, n = 5), inhibitors of the basolateral membrane Na^+-K^+-Cl^- cotransporter.

The magnitude of the cation current activated during cell shrinkage was dependent on the concentration of Cl^- in the external solution. Replacement of external Cl^- with equimolar amounts of the sodium salt of I^- or Br^- produced neither a shift in current reversal potential nor a decrease in the magnitude of the shrinking-induced current (n = 3). However, partial replacement of Cl^- by the impermeant anion aspartate decreased the magnitude of the current without an accompanying change in reversal potential (Fig. 4). Similar results were obtained when glutamate was used as the impermeant anion substitute. When 105 mM NaCl was replaced with sodium glutamate, the swelling-induced current magnitude was reduced by 75 ± 10% at −90 mV and 85 ± 14% at +50 mV (n = 4). In order to demonstrate that the impermeant anion substitutes were not simply inhibiting the cation conductance, we sequentially exposed cells to solutions made hypertonic by either the addition of sucrose or NMDG-aspartate in the presence of 140 mM NaCl. In three cells, there was no significant difference in the increase of current induced by the hypertonic sucrose solutions (906 ± 339 pA at 50 mV) as compared to the change in current induced by the hypertonic NMDG-aspartate solutions (660 ± 278 pA). Therefore, the reduction in shrinking-induced current amplitude, which paralleled the reduction in external Cl^- concentration, was not due to

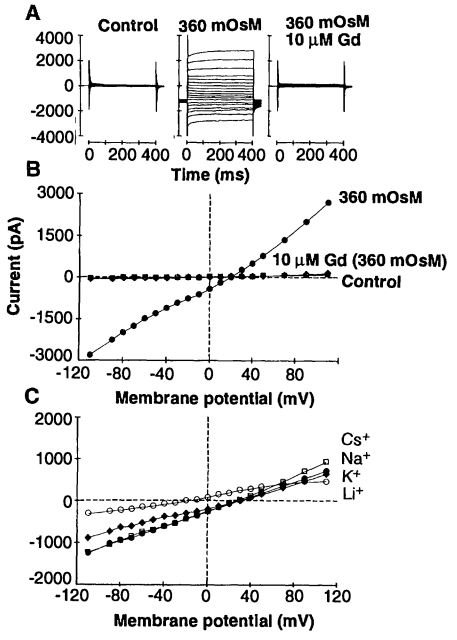

Fig. 2. Osmotic pressure–sensitive current and its selectivity among cations. (**A**) Whole-cell current recordings before and after sequential exposure of a voltage-clamped cell to hypertonic solutions (360 mOsM), with or without gadolinium (Gd) (10 μM). The cell was maintained at a holding potential of −40 mV and stepped to potentials between −110 and +100 mV in 10-mV intervals. Control current amplitude in the standard 280-mOsM bathing solution was 153 ± 32 pA at 50 mV (n = 29). (**B**) Corresponding *I-V* relation for the cell in (A) measured 50 ms after the onset of the voltage pulse. The pipette and bath solutions were similar to those described in Fig. 1. (**C**) *I-V* relations of the shrinking-induced current obtained from a cell in which the bathing solution was sequentially changed to solutions in which Na^+ had been replaced with the chloride salt of Cs (■), K (◆), and Li (○). Filled circles represent *I-V* data obtained in the presence of extracellular Na^+.

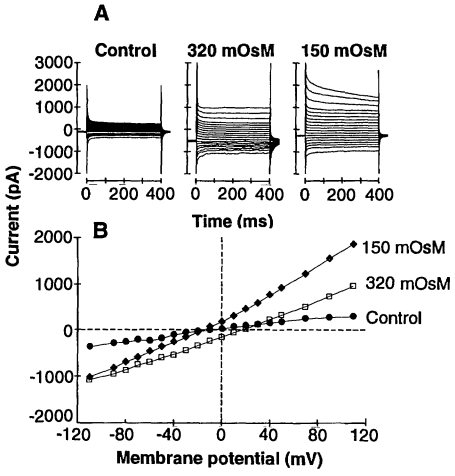

Fig. 3. Separation of conductances activated in either hypertonic or hypotonic bathing solutions. (**A**) Current recordings before and after exposure of a cell to hypertonic (320 mOsM) or hypotonic (150 mOsM) solutions. Solutions were similar to those described in Fig. 1. (**B**) Corresponding *I-V* relations for currents in (A). Note that the zero current potential was +17 mV [theoretical Na^+ equilibrium potential (E_{Na}) was +31 mV] after exposure of the cell to a hypertonic solution. The zero current potential shifted to −15 mV (theoretical E_{Cl} was −20 mV) after subsequent exposure of the cell to a hypotonic solution.

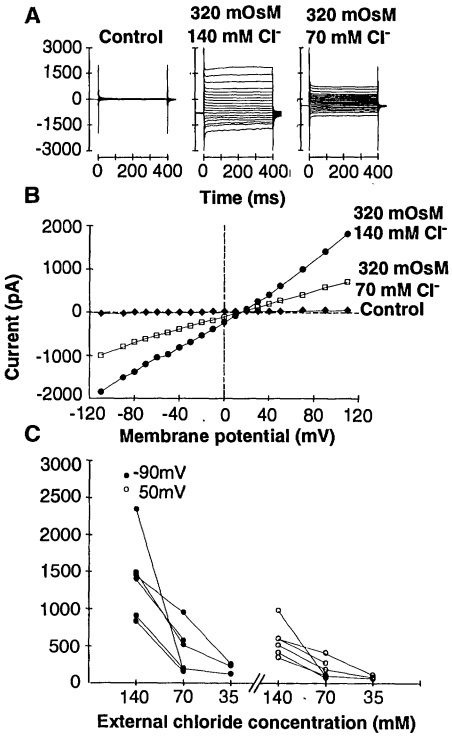

Fig. 4. Anion dependence of the shrinking-induced cation conductance. (**A**) Whole-cell current recordings before and after exposure of a cell to hypertonic extracellular solutions containing varying concentrations of Cl^-. (**B**) Corresponding *I-V* relations for the currents in (A). (**C**) Summary of effect of partial replacement of external Cl^- by aspartate on the shrinking-induced current. Peak current amplitude (at −90 and +50 mV) is plotted as a function of external Cl^- concentration (each experiment is represented by two or three connected points).

current inhibition by the impermeant anion substitute. On the other hand, changes in the intracellular Cl^- concentration produced by the exchange of solutions in the pipette (9) failed to alter the magnitude of the shrinking-induced current or the reversal potential ($n = 5$). These results suggest the presence of an external anion binding site that may act as a modulator for the cation conductance.

We have characterized in airway epithelial cells a nonselective cation conductance that is activated during shrinkage of cells and is dependent on the concentration of external Cl^-. Although nonselective cation channels have been characterized in a number of epithelial tissues, including those of rat pancreatic cells (*10*) and human nasal and sweat gland epithelial cells (*11*), the physiological role of the conductance has not been elucidated experimentally. The nonselective cation channels previously described were characterized by a variable Ca^{2+} dependence; the nonselective cation conductance observed in our study was not Ca^{2+}-dependent. In our experiments, the concentration of intracellular free Ca^{2+} was buffered to 20 nM, which is several orders of magnitude lower than the concentration used in the earlier studies. Activation of the shrinking-induced currents was also observed when cells were bathed in Ca^{2+}-free solutions ($n = 4$). Thus, the Cl^--dependent cation conductance may be different from the nonselective cation conductances described in earlier studies.

The Cl^- efflux induced during cell swelling (*2, 3*) and the Na^+ influx through the nonselective cation conductance activated during shrinkage appear to be part of a volume regulatory response that offsets anisosmotically induced swelling and shrinkage of cells, respectively. The anion dependence of the nonselective cation conductance provides a means for the regulation of Na^+ entry or the inward movement of osmolytes during RVD. When cells are exposed to a hypotonic environment (low external Cl^-), the cation conductance is inactivated and the anion conductance becomes maximally activated, thereby facilitating an efflux of osmotically active particles and consequently a reduction in cell volume. Conversely, when cells are exposed to a hypertonic environment, maximal activation of the cation conductance and the reciprocal inactivation of the anion conductance would facilitate an influx of osmotically active particles and the accompanying increase in cell volume. Thus, the two conductances seem to have complementary functions in cell volume regulation (*12*). The unusual dependence of the nonselective cation conductance on the extracellular Cl^- concentration may be a mechanism whereby cells under osmotic stress control Na^+ entry in such a way that would allow for the tight regulation of Cl^- secretion and Na^+ reabsorption.

REFERENCES AND NOTES

1. M. E. Chamberlin and K. Strange, *Am. J. Physiol.* **257**, 159 (1989).
2. R. T. Worrell *et al.*, *ibid.* **256**, 1111 (1989); A. G. Butt *et al.*, *ibid.* **258**, 630 (1990); A. Rothstein and E. Mack, *ibid.*, p. 827; C. K. Solc and J. J. Wine, *ibid.* **261**, 658 (1991).
3. H. C. Chan *et al.*, *ibid.* **262**, 1273 (1992).
4. Y. Okada and A. Hazama, *News Physiol. Sci.* **4**, 238 (1989); M. M. Y. Wong and J. K. Foskett, *Science* **254**, 1014 (1991).
5. The methods for enzymatically dispersing and plating human turbinate cells and for whole-cell voltage clamping have been described by Chan *et al.* (*3*). We obtained access to the cell interior by applying a pulse of negative pressure to the pipette interior with a success rate of approximately 80%. The success rate for the formation of a stable whole-cell recording was dramatically increased when pipettes were not coated with the insulating resin Sylgard (Dow Corning, Midland, MI). High-resistance seal formation was apparently inhibited by trace amounts of resin that contaminated the electrode tip even after extensive fire polishing. Whole-cell currents in this study were not leak- or capacity-corrected. Currents recorded under experimental conditions (after changes in solution osmolarity) were not corrected by subtracting control currents.
6. Both standard pipette and bath solutions had an osmolarity of 280 mOsM. A bath solution with an osmolarity greater than or less than 280 mOsM was considered either hypertonic or hypotonic with reference to the standard pipette solution, respectively.
7. The pipette solution contained 40 mM NaCl, 100 mM NMDG-aspartate, 10 mM Hepes, 5 mM EGTA, 0.5 mM $CaCl_2$ (20 nM free Ca^{2+}), and 1 mM $MgCl_2$, pH 7.2. The bath solution contained 140 mM NaCl, 10 mM Hepes, 2 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.2.
8. H. C. Chan *et al.* (*3*). Bumetanide and furosemide were dissolved in dimethyl sulfoxide (DMSO) stock solutions and used at a final DMSO concentration of 0.1%.
9. Cells were dialyzed with intracellular (pipette) solutions containing 20, 40, or 80 mM Cl^-. Pipette solutions were exchanged according to the method of M. Soejima and A. Noma [*Pfluegers Arch.* **400**, 424 (1984)].
10. H. Gögelein and B. Pfannmuller, *Eur. J. Physiol.* **413**, 287 (1989); M. A. Gray and B. E. Argent, *Biochim. Biophys. Acta* **1029**, 33 (1990).
11. M. Jorissen *et al.*, *Eur. J. Physiol.* **415**, 617 (1990); M. E. Krouse *et al.*, *Am. J. Physiol.* **257**, 129 (1989).
12. Our studies do not address the role of K^+ conductances in volume regulation. Because the role of K^+ channel activation has been characterized previously (*2, 3*), we omitted K^+ from our solutions to simplify the present investigation.

13. Supported by the Cystic Fibrosis Foundation. We thank M. Haas for help in cell preparation, B. Jow for technical assistance, and H. Fozzard for helpful comments on the manuscript.

11 February 1992; accepted 14 May 1992

Mechanistic Aspects of Signaling Through Ras in NIH 3T3 Cells

Ke Zhang, Alex G. Papageorge, Douglas R. Lowy

Serum and growth factors can increase the proportion of Ras in the active guanosine triphosphate (GTP)-bound form. Growth factors might stimulate guanine nucleotide exchange or decrease the activity of the guanosine triphosphatase–activating proteins GAP and neurofibromin (NF1). In NIH 3T3 cells that overexpress the mutant Ras protein His^{116}, which releases bound guanine nucleotide at a constitutively high rate and retains sensitivity to GAP and NF1, the proportion of GTP bound to the His^{116} protein was not altered by serum or platelet-derived growth factor. However, these mitogens increased the proportion of Ras in the GTP-bound form in cells that overexpressed control Ras proteins with a normal intrinsic rate of guanine nucleotide release. The amount of GTP-bound His^{116} or control Ras proteins was higher in cells at low density than in cells at high density, which have more GAP-like activity. The lower proportion of GTP-bound Ras in NIH 3T3 cells at high density may result from increased GAP-like activity. By contrast, serum and platelet-derived growth factors appear to stimulate guanine nucleotide exchange.

The proteins encoded by the *ras* genes are essential for the transduction of diverse extracellular signals to intracellular targets (*1–3*). The Ras proteins bind guanine nucleotides with high affinity, have intrinsic guanosine triphosphatase (GTPase) activity, and cycle between an active, GTP-bound state and an inactive, guanosine diphosphate (GDP)-bound state (*4, 5*). The proportion of GTP-Ras appears to be determined by two reactions: (i) the rate of guanine nucleotide exchange, which because of the much higher intracellular concentration of GTP than GDP will tend to activate the protein by favoring the GTP-bound form, and (ii) the rate of GTP hydrolysis, which converts GTP-bound Ras to the inactive, GDP-bound form.

Both reactions appear to be enzymatically regulated. GAP (GTPase activating protein) and neurofibromin, the product of *NF1* (the gene that is affected in von Recklinghausen's neurofibromatosis), ac-

Laboratory of Cellular Oncology, Building 37, Room 1B-26, National Cancer Institute, Bethesda, MD 20892.