

Extracellular Hypotonicity Increases Na,K-ATPase Cell Surface Expression *via* Enhanced Na⁺ Influx in Cultured Renal Collecting Duct Cells

MANLIO VINCIGUERRA,* SERGE ARNAUDEAU,[†] DAVID MORDASINI,*
MARTINE ROUSSELOT,* MARCELLE BENS,[‡] ALAIN VANDEWALLE,[‡]
PIERRE-YVES MARTIN,* UDO HASLER,* and ERIC FERAILLE*

*Service de Néphrologie, Fondation pour Recherches Médicales, Geneva, Switzerland; [†]Department of Physiology, University of Geneva Medical Center, Geneva, Switzerland; and [‡]INSERM U478, Faculté de Médecine Xavier Bichat, Paris, France

Abstract. In the renal collecting duct (CD), the Na,K-ATPase, which provides the driving force for Na⁺ absorption, is under tight multifactorial control. Because CD cells are physiologically exposed to variations of interstitial and tubular fluid osmolarities, the effects of extracellular anisotonicity on Na,K-ATPase cell surface expression were studied. Results show that hypotonic conditions increased, whereas hypertonic conditions had no effect on Na,K-ATPase cell surface expression in confluent mpkCCD_{c14} cells. Incubating cells with amphotericin B, which increases [Na⁺]_i, under isotonic or anisotonic conditions, revealed that Na,K-ATPase recruitment to the cell surface was not directly related to variations of cell volume and osmolarity. The effects of amphotericin B and extracellular hypotonicity were not additive, and both were prevented by

protein kinase A and proteasome inhibitors, suggesting a common mechanism of action. In line with this hypothesis, extracellular hypotonicity induced a sustained stimulation of the amiloride-sensitive short-circuit current, indicating increased Na⁺ influx through the apical epithelial Na⁺ channel. Moreover, inhibiting apical Na⁺ entry by amiloride, a blocker of epithelial Na⁺ channel, or incubating cells in Na⁺-free medium prevented the cell surface recruitment of Na,K-ATPase in response to extracellular hypotonicity. Altogether, these findings strongly suggest that extracellular hypotonicity stimulates apical Na⁺ influx leading to increased [Na⁺]_i, protein kinase A activation, and recruitment of Na,K-ATPase units to the cell surface of mpkCCD_{c14} cells.

In the mammalian kidney, water and solute excretion are tightly controlled to maintain body fluid compartment homeostasis. In the collecting duct (CD), which is the site of fine tuning of Na⁺ balance, principal cells are responsible for Na⁺ reabsorption, whereas intercalated cells are involved in acid-base secretion. In principal cells, Na⁺ enters via the luminal epithelial Na⁺ channel (ENaC) and is extruded by basolaterally located Na,K-ATPase, which provides the driving force for vectorial Na⁺ transport (1). The Na,K-ATPase is under multifactorial control including hormones, paracrine factors, intracellular Na ([Na]_i), and extracellular osmolarity (1–3). Long-term regulation of the enzyme relies mainly on altered subunit expression, whereas short-term control is mediated by changes in enzymatic turnover and/or redistribution between cell surface and intracellular compartments (1–3).

Mammalian CD cells are physiologically exposed to variations in both interstitial and luminal osmolalities, which may alter the Na⁺ transport by principal cells. Exposure of renal amphibian A6 cells, a model of mammalian principal cells, to extracellular hypotonicity was shown to stimulate Na⁺ influx through ENaC (4–6). Hypotonic cell swelling is associated with a stimulation of the Na⁺-pump current in both renal A6 cells (7) and cardiac myocytes (8). In addition, hypotonic conditions recruit active Na,K-ATPase units to the cell surface in isolated rabbit cortical CD (CCD) (9). It remains to be determined whether this process relies on the translocation of an intracellular pool of Na,K-ATPase units or alternatively on the activation of silent plasma membrane Na⁺ pumps.

We investigated the mechanism of control of Na,K-ATPase by extracellular tonicity using cultured mouse principal collecting duct mpkCCD_{c14} cells, a cell line derived from microdissected CCD of an SVPK/Tag transgenic mouse (10). These transgenic mice carry the SV40 large T antigen gene (Tag) under the control of the promoter of the L-type pyruvate kinase (PK) gene and of SV40 early enhancer (SV) (11). The mpkCCD_{c14} cells retain expression of Na⁺ and water transporters that are specific for CD principal cells, such as ENaC and aquaporin-2, as well as transepithelial Na⁺ transport controlled

Received April 29, 2004. Accepted July 7, 2004.

Correspondence to Dr. Eric Feraille, Service de Néphrologie, Fondation pour Recherches Médicales, 64 rue de la Roseraie, CH-1211 Geneva 4, Switzerland. Phone: 41-22-382-38-37; Fax: 41-22-347-59-79; E-mail: Eric.Feraille@medecine.unige.ch

1046-6673/1510-2537

Journal of the American Society of Nephrology

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DOI: 10.1097/01.ASN.0000139931.81844.10

by aldosterone and vasopressin, and represent a valuable experimental model (12–14). The results of this study demonstrate that Na,K-ATPase cell surface expression is modulated by Na⁺ influx independent of cell volume variation in mpkCCD_{c14} cells.

Materials and Methods

Cell Culture

The mpkCCD_{c14} cells (passages 20 to 25) were grown in defined medium (DM; DMEM:Ham's F₁₂ 1:1 vol/vol, 60 nM sodium selenate, 5 μg/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml EGF, 5 μg/ml insulin, 20 mM D-glucose, 2% [vol/vol] FCS, and 20 mM HEPES, pH 7.4) at 37°C in 5% CO₂/95% air atmosphere. Experiments were performed on confluent cells seeded on polycarbonate filters (Transwell, 0.4-μm pore size, 1 cm² growth area; Corning Costar, Cambridge, MA). Cells were kept for 6 to 8 d in DM and then placed in serum-free, hormone-deprived medium 24 h before experiments. For experiments, cells were preincubated for 30 min at 37°C with various isotonic incubation solutions that contained either 140 mM NaCl or 240 mM sucrose, supplemented or not with drugs as described in Results and figure legends. Afterward, cells were incubated for an additional hour at 37°C in the same incubation solutions to which 1 μg/ml amphotericin B was added or after apical and basal replacement of the isotonic incubation medium by an equivalent volume of hypotonic (200 mOsm/L) or hypertonic (500 mOsm/L) incubation solution. The final composition of the solutions used is given in Table 1.

Measurement of Cell Surface Na,K-ATPase

Cell surface Na,K-ATPase was determined on cultured mpkCCD_{c14} cells as described previously (12,13) using EZ-Link sulfosuccinimidobiotin (Sulfo NHS-S-S-Biotin; Pierce, Rockford, IL) to label cell surface proteins. After lysis in homogenizing buffer (HB; 2 mM EDTA, 2 mM EGTA, 20 μg/ml leupeptin, 1 μg/ml aprotinin, 30 mM

NaF, 30 mM Na pyrophosphate, 1 mM PMSF, 1 mM AEBBSF, 0.1% [wt/vol] SDS, 1% [vol/vol] Triton X-100, and 20 mM Tris HCl [pH 7.4]), equal amounts of protein were precipitated with streptavidin-agarose beads (Immunopure immobilized streptavidin; Pierce) diluted in an antiprotease-supplemented Tris Lysis Buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 20 μg/ml leupeptin, and 1 μg/ml aprotinin). After three washes with TLB and one with 10 mM Tris-HCl (pH 7.4), samples were resuspended in Laemmli's buffer (15) and processed for 7% SDS-PAGE, and proteins were electrotransferred to polyvinylidene difluoride membranes (Immobilion-P; Millipore, Waters, MA). The Na,K-ATPase α-subunit was then detected with a polyclonal antibody (dilution 1:10000) raised against the rat enzyme (16). The α-transferrin receptor was detected with an mAb (dilution 1:2000; Zymed, San Francisco, CA), and E-cadherin was detected with a polyclonal antibody (dilution 1:5000; Sigma, St. Louis, MO). The protein bands revealed by chemiluminescence (Super Signal Substrate; Pierce) were quantified using a video densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA), and the results were expressed as percentage of control.

Cell Volume Analysis

The mpkCCD_{c14} cells, grown on polycarbonate filters or glass coverslips, were rinsed with PBS and incubated for 30 min at 37°C in culture medium that contained the fluorescent dye calcein-AM (5 μM diluted from a 10-mM stock solution in DMSO; Molecular Probes Europe, Leiden, Netherlands). Dye loading was terminated by rinsing with PBS; cells were then placed in an open perfusion chamber on a stage of an inverted microscope (Zeiss Axiovert 200M; Carl Zeiss AG, Feldbach, Switzerland), and prewarmed (37°C) incubation solutions were superfused with a peristaltic pump (Dynamax, Emeryville, CA). For improving measurements, fluorescence changes were monitored using a spinning disk confocal microscope, which better preserves living cells than a laser scanning confocal microscope and allows normalization of the fluorescence with respect to the thickness

Table 1. Experimental solutions^a

	Solutions							
	NaCl			Sucrose				
	Iso	Hypo	Hyper	Iso	Iso (Na ⁺ free)	Hypo	Hypo (Na ⁺ free)	Hyper
NaCl	140	90	240	40	—	40	—	40
NaHCO ₃	4	4	4	4	—	4	—	4
KHCO ₃	—	—	—	—	4	—	4	—
NaH ₂ PO ₃	0.20	0.20	0.20	0.20	—	0.20	—	0.20
Na ₂ HPO ₃	0.15	0.15	0.15	0.15	—	0.15	—	0.15
KH ₂ PO ₃	—	—	—	—	0.20	—	0.20	—
K ₂ HPO ₃	—	—	—	—	0.15	—	0.15	—
KCl	5	5	5	5	40	5	40	—
MgSO ₄	1	1	1	1	1	1	1	1
Glucose	5	5	5	5	5	5	5	5
CaCl ₂	1	1	1	1	1	1	1	1
Sucrose	—	—	—	240	240	120	120	420
HEPES	20	20	20	20	20	20	20	20
Osmolality	305	215	505	335	345	235	245	535

^a Iso, isosmotic; Hypo, hyposmotic; Hyper, hyperosmotic. Final concentrations are given in mmol/L and osmolality in mOsm/kg H₂O. The pH of all solutions is 7.4.

of the optical slice, improving the accuracy of single-emission dyes. The 488-nm line from a 2.5-W Kr/Ar water-cooled ion laser in multiline mode (Innova 70C Spectrum; Coherent, Santa Clara, CA) was selected for calcein excitation by the use of an Acousto-Optical Tunable Filter (AOTF; Visitech International, Sunderland, UK). The AOTF output was coupled by a single-mode fiber optic (Oz Optics, Ltd., Corp, Ontario, Canada) to a Yokogawa spinning disk confocal scan-head (QLC100; Visitech International) mounted on the inverted microscope. Images were collected by a 40×0.75 NA Achromatic water immersion objective (Carl Zeiss AG) and captured with a 12-bit TE/CCD interlined Coolsnap HQ Photometrics camera (Roper Scientific, Trenton, NJ). The green emission from calcein was acquired using a D525/50m emission filter (Chroma Technology Corp., Brattleboro, VT). Wavelength selection with the AOTF, camera control, and all of the motorized functions of the microscope were controlled for image acquisition by Metamorph/Metafluor 5.0 software (Universal Imaging, West Chester, PA). The same software was used off-line for image analysis, photo-bleaching correction, and curve fitting. Because recorded fluorescence in a given cytoplasmic area is proportional to the intracellular concentration of calcein, we determined cell volume changes by measurement of variations of calcein fluorescence, as described previously (17). The results were expressed as a percentage of the control period fluorescence intensity.

Electrophysiologic Studies

Confluent mpkCCD_{c14} cells that were grown on Snapwell filters (0.4- μ m pore size, 12-mm diameter; Corning Costar) were transferred to a Ussing chamber, and short-circuit current (I_{sc}) was measured under voltage clamp (0 mV) using dual silver-silver chloride electrodes connected to a VCC MC6 voltage-clamp apparatus (Physiologic Instruments, San Diego, CA). Cells were equilibrated at 37°C for 30 min in symmetric isotonic (300 mOsm/L) sucrose buffer that contained 40 mM Na⁺ (Table 1) and bubbled with 5% CO₂. Isotonic sucrose buffer was then replaced by hypotonic sucrose buffer (200 mOsm/L and 40 mM Na⁺) using a peristaltic pump, and cells were maintained for 60 min under symmetric hypotonic conditions. Then, isotonic conditions were reestablished by exchanging the hypotonic sucrose buffer with isotonic sucrose buffer. After 30 min of equilibration, 10⁻⁶ M amiloride was added to the apical side of the chamber to measure the amiloride-resistant I_{sc} . The amiloride-sensitive I_{sc} reflecting ENaC-mediated Na⁺ transport was calculated as the total I_{sc} minus the amiloride-resistant I_{sc} . By convention, positive I_{sc} corresponded to a flow of positive charges from the basal to the apical solution. Results were expressed as μ A/cm².

Statistical Analyses

Results are given as means \pm SEM from n independent experiments. Each experiment was performed on cultured cells from the same passage. Statistical analysis of Na,K-ATPase α -subunit immunoreactivity was done using the Mann-Whitney U test or the Kruskal-Wallis test for comparison of two or more than two groups, respectively. Statistical analysis of I_{sc} was done using t test for paired data. $P < 0.05$ was considered significant.

Results

Effect of Extracellular Anisotonicity on Na,K-ATPase Cell Surface Expression

We first assessed the influence of extracellular tonicity on cell volume and Na,K-ATPase cell surface expression. Incubation of confluent mpkCCD_{c14} cells in a hypotonic medium

(200 mOsm/L) induced a rapid decrease in fluorescence intensity from calcein-loaded cells corresponding to a $24.5 \pm 5.5\%$ increase in cell volume (Figure 1A). After the hypotonic medium was replaced by an isotonic medium (not shown), fluorescence intensity returned toward baseline levels, indicating that the decrease in fluorescence signal was not due to a dye leakage but rather to intracellular calcein dilution consecutive to cell swelling. Incubation of mpkCCD_{c14} cells for 1 h at 37°C in hypotonic medium increased Na,K-ATPase cell surface expression by $\sim 30\%$ as compared with cells that were incubated under isotonic conditions (Figure 1B). A similar increase of Na,K-ATPase cell surface expression was observed when extracellular NaCl was replaced by sucrose (Table 1, Figure 1B, right). Therefore, Na,K-ATPase was recruited to the cell surface independent of variations of ionic strength. As expected, the fluorescence intensity of calcein-loaded cells increased after exposure to a hyperosmotic medium (500 mOsm/L), indicating a $20.1 \pm 2.0\%$ decrease in cell volume (Figure 1C). In contrast to the observations made in cells that were incubated under hypotonic conditions, hypertonic NaCl or sucrose medium (Table 1) did not significantly alter Na,K-ATPase cell surface expression (Figure 1D).

The question arises whether cell swelling controls Na,K-ATPase expression to the cell surface. Using mpkCCD_{c14} cells, we showed that amphotericin B, which forms artificial pores that enhance Na⁺ and K⁺ plasma membrane permeabilities (18), increases Na,K-ATPase cell surface expression in the presence of physiologic concentrations of extracellular Na⁺ (19). Because amphotericin B induces cell swelling in MDCK cells (17), we tested whether variations of cell volume may account for the Na⁺ ionophore-induced Na,K-ATPase recruitment. For this purpose, mpkCCD_{c14} cells were preincubated in isotonic NaCl medium (Table 1) for 1 h at 37°C and then for an additional hour in isotonic or hypotonic NaCl medium (200 mOsm/L) with or without 1 μ g/ml amphotericin B. Figure 2A and 3A show that under isotonic conditions, amphotericin B did not alter calcein fluorescence intensity, indicating the absence of a detectable variation of cell volume. In the absence of amphotericin B, incubation of cells with hypotonic medium (200 mOsm/L) decreased calcein fluorescence, reflecting cell swelling (Figure 1A), whereas calcein fluorescence slightly increased in cells that were challenged with both amphotericin B and hypotonic medium (Figure 2B). The latter increase in calcein fluorescence most likely reflected a moderate cell shrinkage ($9.9 \pm 1.5\%$) consecutive to the diffusion of ions and accompanying water through amphotericin B pores from the cytosol to the less concentrated extracellular medium (18). We next analyzed the combined effects of extracellular hypotonicity and amphotericin B permeabilization on Na,K-ATPase cell surface expression to determine whether they potentially share a common mechanism of action despite dissimilar effects on cell volume. Amphotericin B and extracellular hypotonicity increased Na,K-ATPase cell surface expression to a similar extent and in a nonadditive manner (Figure 2C). These results suggest that both stimuli activate similar or convergent signaling pathways.

To investigate further the relationship between extracellular

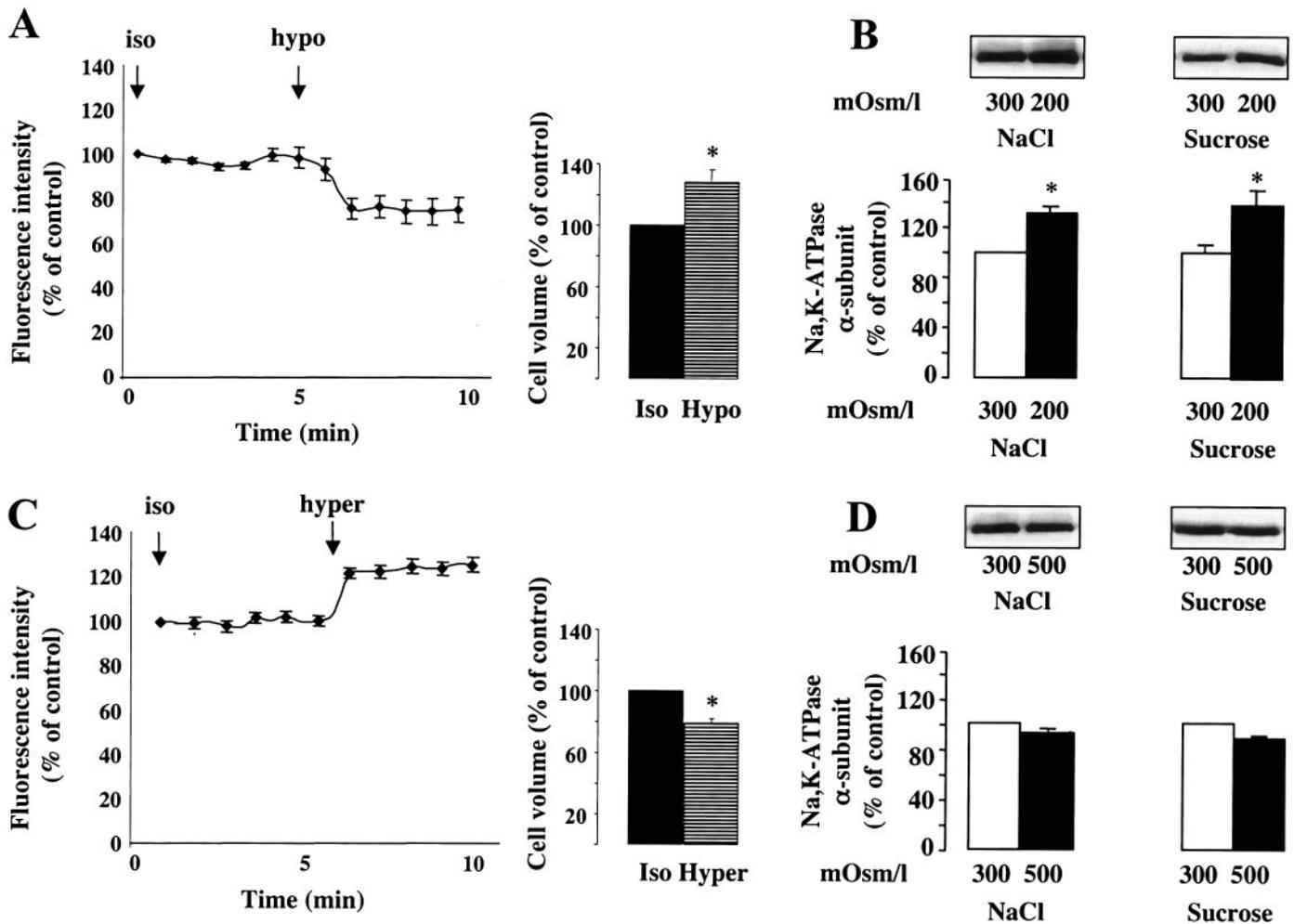


Figure 1. Effect of extracellular hypo- or hypertonicity on cell volume and Na,K-ATPase cell surface expression. (A and C) Confluent mpkCCD_{c14} cells that were grown on glass coverslips were loaded with calcein-AM and mounted in an open perfusion chamber placed on a stage of an inverted microscope. After a 30-min equilibration period in isotonic incubation solution (300 mOsm/L; iso), cells were superfused with either hypotonic (200 mOsm/L; hypo; A) or hypertonic (500 mOsm/L; hyper; C) incubation solutions and fluorescence intensity was recorded. The graph illustrates the changes in calcein fluorescence (line) and the maximal variation of cell volume (bars) expressed as a percentage of baseline. Results are means \pm SEM from three separate experiments. (B and D) Confluent mpkCCD_{c14} cells that were grown on polycarbonate filters were first preincubated in the presence of isotonic (300 mOsm/L) NaCl- or sucrose-containing incubation solution (Table 1) for 1 h at 37°C and then incubated under isotonic, hypotonic (B), or hypertonic conditions (D) for 1 h at 37°C. The Na,K-ATPase α -subunit was detected by Western blotting performed after biotinylation and streptavidin precipitation of cell surface proteins. (Top) Representative immunoblots showing Na,K-ATPase cell surface expression. (Bottom) The densitometric values of labeled Na,K-ATPase α -subunit bands were expressed as a percentage of the optical density value of cells that were incubated in isotonic medium (100%). Results are means \pm SEM from six separate experiments. * $P < 0.05$ versus 300 mOsm/L values.

tonicity, Na⁺ permeability, and Na,K-ATPase cell surface expression, we examined the effect of extracellular hypertonicity on the amphotericin B-induced increase in Na,K-ATPase cell surface expression. For this purpose, mpkCCD_{c14} cells were preincubated in NaCl isotonic medium (Table 1) for 1 h at 37°C and incubated for an additional hour in isotonic or hypertonic NaCl medium (500 mOsm/L) with or without 1 μ g/ml amphotericin B. In the absence of amphotericin B, extracellular hypertonicity induced cell shrinkage (Figure 1C), whereas the addition of amphotericin B to the hypertonic solution induced a slight decrease in calcein fluorescence, reflecting a moderate cell swelling ($12.2 \pm 1.2\%$; Figure 3B). This

latter effect most likely relies on extracellular ion entry and accompanying water through the membrane pores formed by amphotericin B (18). Adding 200 mOsm/L NaCl or sucrose (corresponding to the 500 mOsm/L hypertonic solution) did not significantly modify Na,K-ATPase cell surface expression (Figure 3C). However, addition of amphotericin B to both hypertonic and isotonic solution increased Na,K-ATPase cell surface expression (Figure 3C). These results indicate that hypertonicity does not modulate the effect of increased Na⁺ influx caused by amphotericin B on Na,K-ATPase recruitment to the cell surface. Altogether, our results demonstrate that the recruitment of Na,K-ATPase to the cell surface of mpkCCD_{c14}

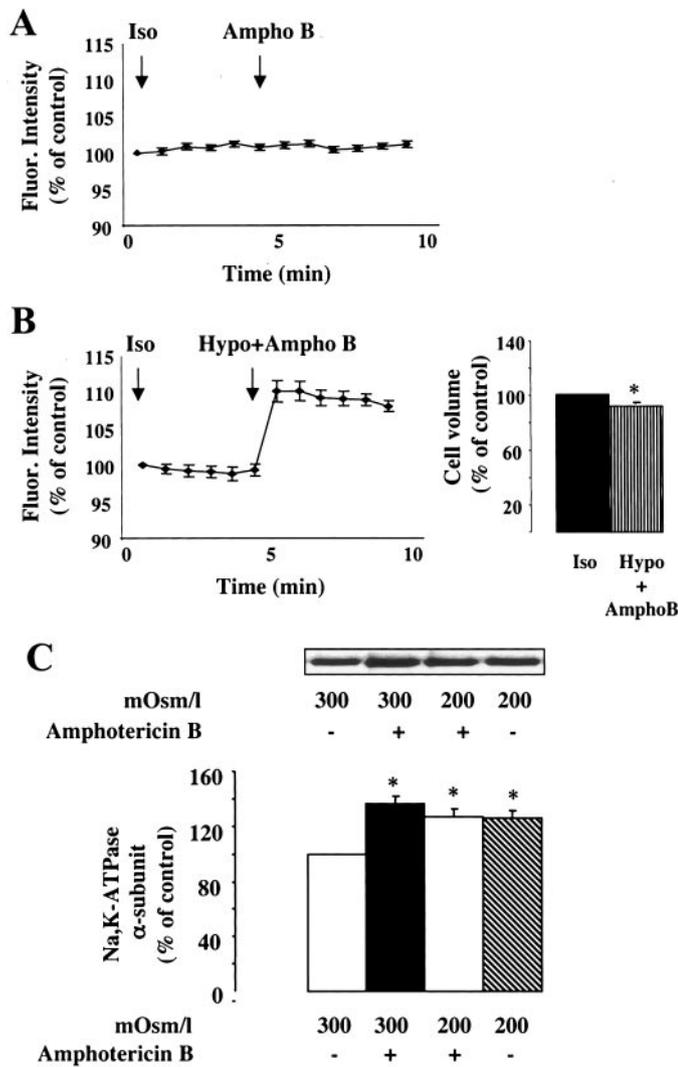


Figure 2. Effect of amphotericin B and extracellular hypotonicity on cell volume and Na,K-ATPase cell surface expression. (A and B) Confluent mpkCCD_{c14} cells that were grown on glass coverslips were loaded with calcein-AM as described in the legend of Figure 1. After equilibration with isotonic incubation solution (300 mOsm/L; iso), cells were superfused with 1 μ g/ml amphotericin B diluted in isotonic (Ampho B; A) or hypotonic (200 mOsm/L; hypo; B) solutions and fluorescence intensity was recorded. The graph illustrates the changes in calcein fluorescence (line) and the maximal variation of cell volume (bars) expressed as a percentage of baseline. Results are means \pm SEM from three separate experiments. (C) Confluent mpkCCD_{c14} cells that were grown on polycarbonate filters were first preincubated in the presence of isotonic (300 mOsm/L) NaCl-containing incubation solution (Table 1) for 1 h at 37°C. Cells were then permeabilized or not with 1 μ g/ml amphotericin B and then incubated with isotonic (300 mOsm) or hypotonic (200 mOsm) solution for 1 h at 37°C. The Na,K-ATPase α -subunit was then detected by Western blotting performed after biotinylation and streptavidin precipitation of cell surface proteins. (Top) Representative immunoblots showing Na,K-ATPase cell surface expression. (Bottom) The densitometric values of labeled Na,K-ATPase α -subunit bands were expressed as a percentage of variations of the optical density value of cells that were incubated in isotonic medium in the absence of amphotericin B (100%). Results are means \pm SEM from eight separate experiments. **P* < 0.05 versus 300 mOsm/L without amphotericin B values.

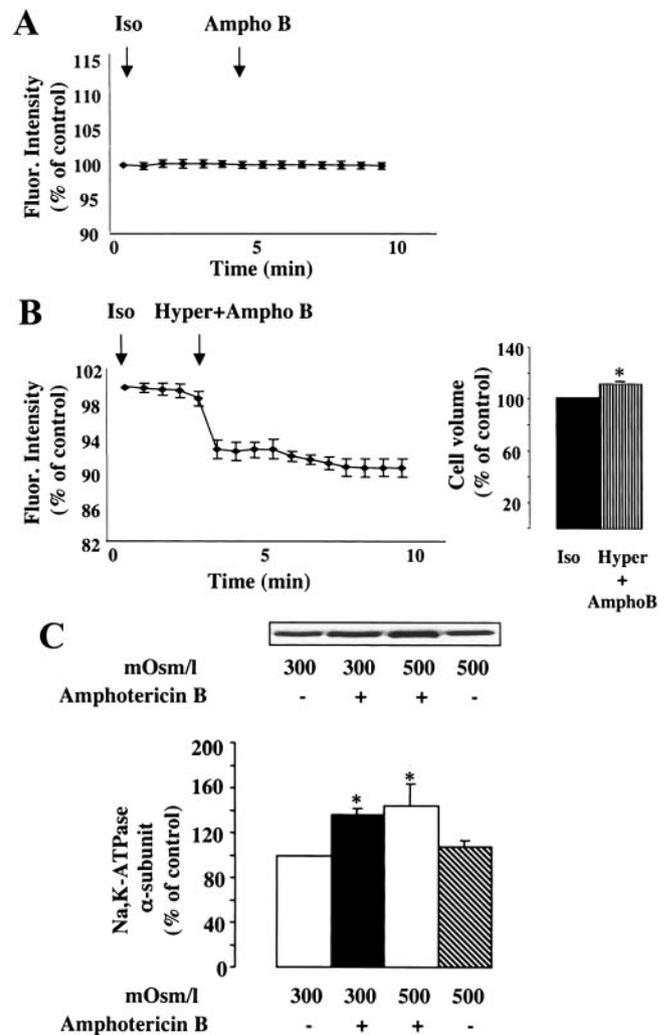


Figure 3. Effect of amphotericin B and extracellular hypertonicity on cell volume and Na,K-ATPase cell surface expression. (A and B) Confluent mpkCCD_{c14} cells that were grown on glass coverslips were loaded with calcein-AM, equilibrated with the isotonic incubation solution (Iso), and superfused with 1 μ g/ml amphotericin B as described in the legend of Figure 2. Amphotericin B was diluted in either isotonic (Ampho B; A) or hypertonic (500 mOsm/L; hyper; B) incubation solution and fluorescence intensity was recorded. The graph illustrates the changes in calcein fluorescence (line) and the maximal variation of cell volume (bars) expressed as a percentage of baseline. Results are means \pm SEM from three separate experiments. (C) Confluent mpkCCD_{c14} cells that were grown on polycarbonate filters were first preincubated in the presence of isotonic (300 mOsm/L) NaCl incubation solution (Table 1) for 1 h at 37°C. Cells were then permeabilized or not with 1 μ g/ml amphotericin B and then incubated under either isotonic (300 mOsm/L) or hypertonic (500 mOsm/L) solution for 1 h at 37°C. The Na,K-ATPase α -subunit was then detected by Western blotting performed after biotinylation and streptavidin precipitation of cell surface proteins. (Top) Representative immunoblots showing Na,K-ATPase cell surface expression. (Bottom) The densitometric values of labeled Na,K-ATPase α -subunit bands were expressed as a percentage of variations of the optical density value of cells that were incubated in isotonic medium in the absence of amphotericin B (100%). Results are means \pm SEM from eight separate experiments. **P* < 0.05 versus 300 mOsm/L without amphotericin B values.

Table 2. Correlation between cell volume variation, Na⁺ entry, and increase in Na,K-ATPase cell surface expression

Medium	Cell Volume Variation	Na ⁺ Entry	Na,K-ATPase Surface Expression
Isotonic NaCl	—	—	—
Hypotonic NaCl	↑	↑	↑
Hypertonic NaCl	↓	—	—
Amphotericin B (1 μg/ml)	—	↑	↑
Hypotonic NaCl + Amphotericin B	↓	↑	↑
Hypertonic NaCl + Amphotericin B	↑	↑	↑

cells is not directly correlated to changes in cell volume (Table 2).

Extracellular Hypotonicity Increases Na,K-ATPase Cell Surface Expression via a Protein Kinase A- and Proteasome-Dependent Pathway

The lack of additive effects of amphotericin B and extracellular hypotonicity on Na,K-ATPase membrane recruitment suggests that both stimuli activate convergent signaling pathways. In CD principal cells, Na,K-ATPase cell surface recruitment induced by amphotericin B-dependent Na⁺ influx is mediated by cAMP-independent PKA activation that requires proteasomal activity (19). To determine whether a similar pathway is involved in the observed hypotonicity-induced increase in Na,K-ATPase cell surface expression, we preincubated mpkCCD_{c14} cells in the absence or presence of 10⁻⁵ M H89, a protein kinase A (PKA) inhibitor, for 1 h at 37°C before exposure or not to a hypotonic (200 mOsm/L) NaCl or sucrose medium with or without H89 for an additional hour. Inhibition of PKA by H89 fully prevented the increase in Na,K-ATPase cell surface expression induced by NaCl or sucrose hypotonic solution (Figure 4). We next investigated whether the hypotonicity-induced increase in Na,K-ATPase cell surface expression also requires proteasomal activity. For this purpose, we preincubated mpkCCD_{c14} cells for 2 h at 37°C in the absence or presence of 10⁻⁶ M of the proteasomal inhibitor lactacystin before exposure or not to hypotonic (200 mOsm/L) NaCl medium with or without lactacystin for an additional hour. Figure 5 shows that lactacystin prevented the hypotonicity-induced Na,K-ATPase recruitment. Similar results were achieved using 10⁻⁵ M MG132, a proteasomal inhibitor that is structurally unrelated to lactacystin (data not shown). Taken together, these results indicate that amphotericin B and extracellular hypotonicity both increased Na,K-ATPase cell surface expression *via* the same mechanism.

Hypotonicity Stimulates the Amiloride-Sensitive I_{SC} in mpkCCD_{c14} Cells

In renal amphibian A6 cells, a model of CD principal cells, extracellular hypotonicity was shown to stimulate transepithelial Na⁺ transport by increasing both the number of ENaC (4–6) present in the apical membrane and Na,K-ATPase activity (7). We therefore determined whether a hypotonic challenge stimulates the amiloride-sensitive I_{SC} in mpkCCD_{c14} cells. Cells were first equilibrated in isotonic sucrose solution

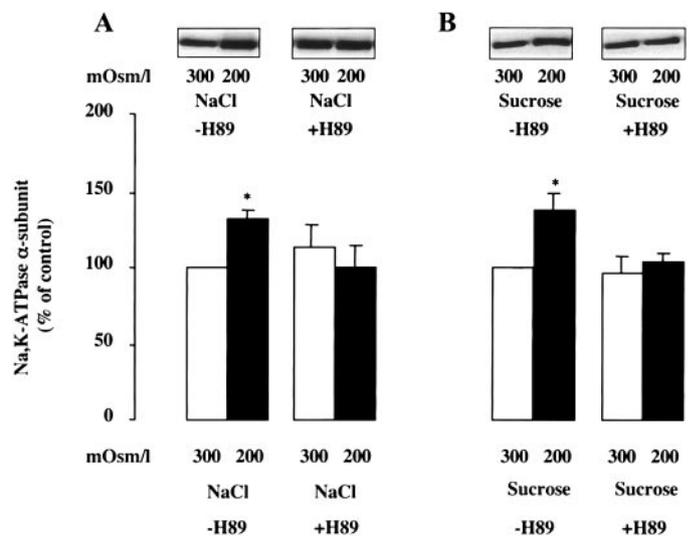


Figure 4. The hypotonicity-induced increase in Na,K-ATPase cell surface expression is dependent on protein kinase A (PKA). Confluent mpkCCD_{c14} cells that were grown on polycarbonate filters were first incubated in the presence of isotonic (300 mOsm/L) NaCl (A) or sucrose (B) incubation solution (Table 1) and with (–) or without (+) 10⁻⁶ M H89, a PKA inhibitor, for 1 h at 37°C. Cells were then incubated under either isotonic or hypotonic (200 mOsm/L) solution supplemented or not with H89 for 1 h at 37°C. Na,K-ATPase α-subunit was then detected by Western blotting performed after biotinylation and streptavidin precipitation of cell surface proteins. (Top) Representative immunoblots showing Na,K-ATPase cell surface expression. (Bottom) The densitometric values of labeled Na,K-ATPase α-subunit bands were expressed as a percentage of the optical density value of cells that were incubated in isotonic medium in the absence of H89 (100%). Results are means ± SEM from five separate experiments. *P < 0.05 versus 300 mOsm/L without H89 values.

(300 mOsm/L) that contained 40 mM Na⁺ (Table 1). Under these conditions, I_{SC} was stable for at least 120 min. After exposure of mpkCCD_{c14} cells to a hyposmotic sucrose solution (200 mOsm/L) in the continuous presence of 40 mM extracellular Na⁺, the amiloride-sensitive I_{SC} gradually increased from 4.8 ± 0.4 to 8.7 ± 0.6 μA/cm² (P < 0.01) during the first 20 min of incubation, and this stimulation was sustained for at least 40 min (Figure 6A). The amiloride-sensitive I_{SC} promptly returned to a nearly basal levels (5.3 ± 0.3 μA/cm²) after reestablishment of isotonic conditions (Figure 6). However, the amiloride-insensitive I_{SC} was similar under isotonic and hypo-

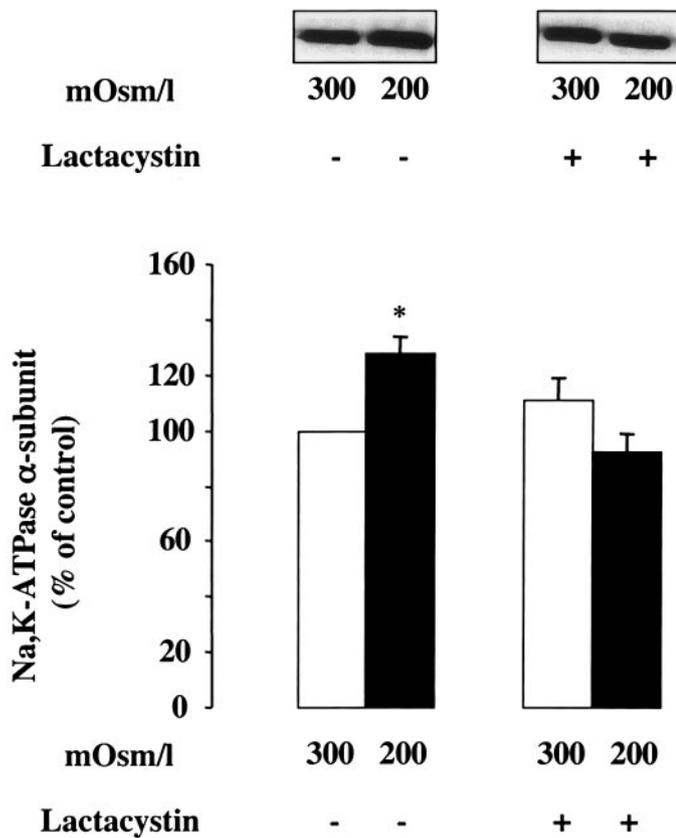


Figure 5. The hypotonicity-induced increase in Na,K-ATPase cell surface expression is dependent on proteasomal activity. Confluent mpkCCD_{c14} cells that were grown on polycarbonate filters were first preincubated in the presence of isotonic (300 mOsm/L) NaCl incubation solution with (–) or without (+) 10^{–6} M lactacystin, a proteasomal inhibitor, for 1 h at 37°C. Cells were then incubated under either isotonic or hypotonic (200 mOsm/L) conditions and in the presence or absence of lactacystin for 1 h at 37°C. Na,K-ATPase α -subunit was then detected by Western blotting performed after biotinylation and streptavidin precipitation of cell surface proteins. (Top) Representative immunoblots showing Na,K-ATPase cell surface expression. (Bottom) The densitometric values of labeled Na,K-ATPase α -subunit bands were expressed as a percentage of the optical density value of cells that were incubated in isotonic medium and in the absence of lactacystin (100%). Results are means \pm SEM from five separate experiments. **P* < 0.05 versus 300 mOsm/L without lactacystin values.

tonic conditions ($0.4 \pm 0.1 \mu\text{A}/\text{cm}^2$). These findings indicate that extracellular hypotonic challenge enhances the apical Na⁺ influx through ENaC and therefore may increase [Na⁺]_i in mpkCCD_{c14} cells.

Increase in Na,K-ATPase Cell Surface Expression in Response to Extracellular Hypotonicity Depends on Apical Na⁺ Entry

Our results showed that extracellular hypotonicity stimulated the amiloride-sensitive *I*_{SC} in mpkCCD_{c14} cells, suggesting that increased Na,K-ATPase cell surface expression in response to extracellular hypotonic challenge relies on enhanced Na⁺ influx through ENaC. To test this hypothesis, we

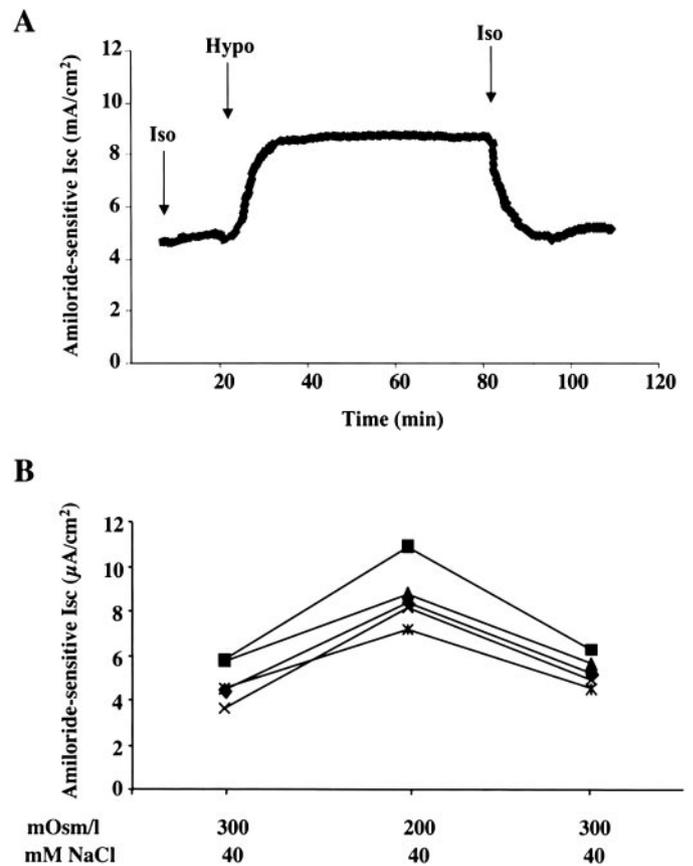


Figure 6. Hypotonicity stimulates the amiloride-sensitive short-circuit current (*I*_{SC}) in mpkCCD_{c14}. Confluent mpkCCD_{c14} cells that were grown on polycarbonate filters (Snapwell) were transferred to a Ussing chamber, and *I*_{SC} was measured under voltage clamp (0 mV) as described in Materials and Methods. Cells were first equilibrated for 30 min at 37°C in the presence of isotonic (300 mOsm/L) sucrose incubation solution and then exposed to hypotonic (200 mOsm/L) sucrose incubation solution for 60 min before reestablishing isotonic conditions. All incubation solutions contained 40 mM Na⁺. (A) Representative recording showing *I*_{SC} as a function of time and extracellular osmolarity. (B) Amiloride-sensitive *I*_{SC} under isosmotic (300 mOsm/L), hyposmotic (200 mOsm/L), and reestablishment of isosmotic condition. Results from five separate experiments are shown.

preincubated mpkCCD_{c14} cells that were grown on filters under isotonic conditions for 30 min at 37°C with or without 10^{–6} M amiloride, an inhibitor of ENaC, before exposure to a hypotonic (200 mOsm/L) extracellular solution with or without amiloride for an additional hour. Amiloride fully prevented the increase in Na,K-ATPase cell surface expression induced by extracellular hypotonicity (Figure 7). Experiments were then performed using a nominally Na⁺-free medium (Table 1) to investigate further the Na⁺ dependence of the hypotonicity-induced recruitment of Na,K-ATPase to the cell surface. Figure 8, A and B, shows that exposure of calcein-loaded cells to hypotonic (200 mOsm/L) sucrose medium induced a similar increase in cell volume in the presence of a residual 40 mM Na⁺ ($23.0 \pm 2.2\%$) or in the nominal absence of Na⁺ ($21.9 \pm$

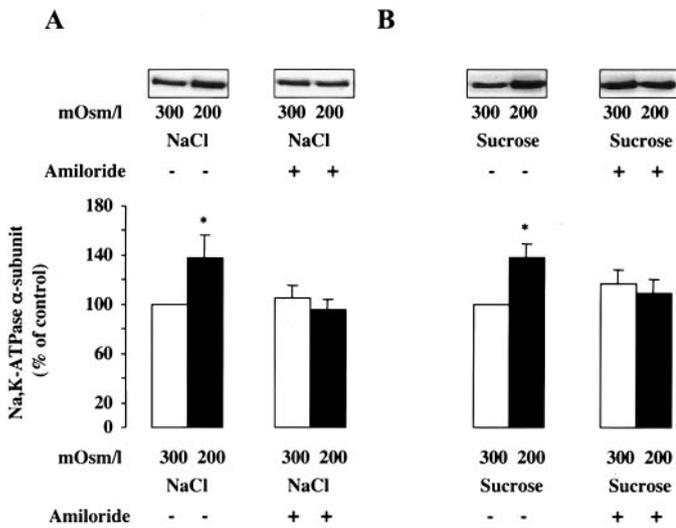


Figure 7. The hypotonicity-induced increase in Na,K-ATPase cell surface expression is dependent on epithelial NaCl channel activity. Confluent mpkCCD_{c14} cells that were grown on polycarbonate filters were first incubated in the presence of isotonic (300 mOsm) NaCl (A) or sucrose (B) incubation solution with (–) or without (+) 10^{–6} M amiloride for 30 min at 37°C. Cells were then incubated in either an isotonic or a hypotonic (200 mOsm/L) solution and in the presence or absence of amiloride for 1 h at 37°C. The Na,K-ATPase α-subunit was detected by Western blotting performed after biotinylation and streptavidin precipitation of cell surface proteins. (Top) Representative immunoblot showing Na,K-ATPase cell surface expression. (Bottom) The densitometric values of labeled Na,K-ATPase α-subunit bands were expressed as a percentage of the optical density value of cells that were incubated in isotonic medium and in the absence of amiloride (100%). Results are means ± SEM from four separate experiments. *P < 0.05 versus 300 mOsm/L without amiloride values.

2.6%). A significant increase in cell surface Na,K-ATPase was still observed when using a hypotonic (200 mOsm/L) solution in which NaCl was substituted by sucrose but still contained a residual 40 mM Na⁺ as compared with that measured using an isotonic (300 mOsm/L) sucrose solution that contained the same concentration of Na⁺ (Figure 8C, left). In contrast, extracellular hypotonicity did not increase Na,K-ATPase cell surface expression when mpkCCD_{c14} cells were incubated in a nominally Na⁺-free solution in which NaCl was substituted by sucrose and in which the remaining 40 mM Na⁺ was substituted by K⁺ (Table 1, Figure 8C, right). The hypotonicity-induced recruitment of Na,K-ATPase was blunted in the absence of Na⁺ despite a similar increase in cell volume and therefore cell surface area, suggesting that Na,K-ATPase is specifically targeted to the cell surface in the presence of extracellular Na⁺. Figure 8D shows that, in agreement with the functional link between Na,K-ATPase and E-cadherin observed in epithelial cells, exposure of mpkCCD_{c14} cells to NaCl hypotonic medium induced a parallel increase of the cell surface expression of both proteins (20). In contrast, the cell surface expression of the ubiquitously expressed and quickly recycling α-transferrin receptor (21) remained unchanged.

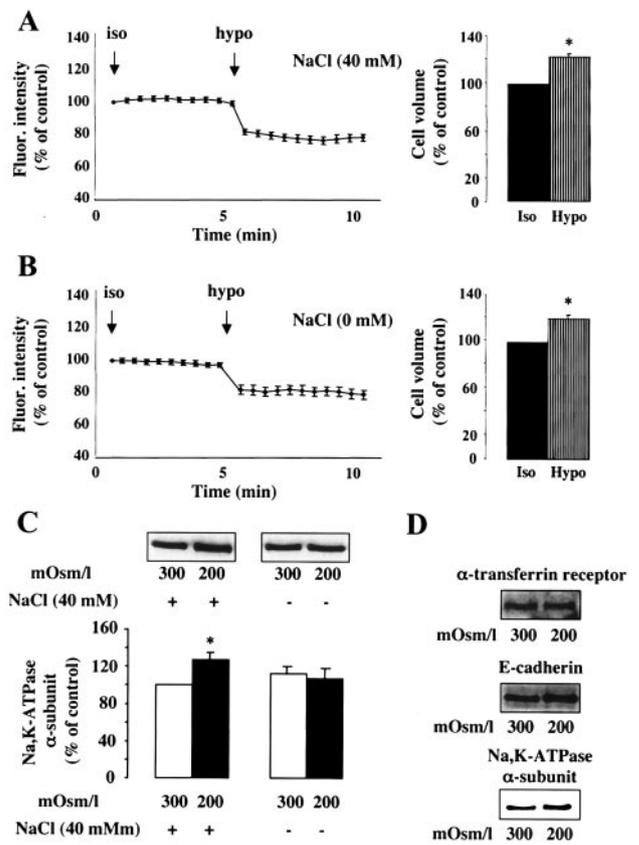


Figure 8. The hypotonicity-induced increase of Na,K-ATPase cell surface expression is dependent on the presence of extracellular sodium. (A and B) Confluent mpkCCD_{c14} cells that were grown on glass coverslips were loaded with calcein-AM and mounted in an open perfusion chamber placed on a stage of an inverted microscope. After a 30-min equilibration period in isotonic sucrose incubation solution (300 mOsm/L; iso), cells were superfused with hypotonic sucrose (200 mOsm/L; hypo) incubation solutions that contained (A) or not (B) 40 mM Na⁺ and fluorescence intensity was recorded. The graph illustrates the changes in calcein fluorescence (line) and the maximal variation of cell volume (bars) expressed as a percentage of baseline. Results are means ± SEM from two separate experiments. (C) Confluent mpkCCD_{c14} cells that were grown on polycarbonate filters were first preincubated in the presence of isotonic (300 mOsm/L) sucrose incubation solution that contained (40 mM) or not (0 mM) Na⁺ (Table 1) for 1 h at 37°C. Cells were then incubated in either an isotonic or a hypotonic (200 mOsm/L) solution and in the presence or absence of Na⁺ for 1 h at 37°C. The Na,K-ATPase α-subunit was detected by Western blotting performed after biotinylation and streptavidin precipitation of cell surface proteins. (Top) Representative immunoblot showing Na,K-ATPase cell surface expression. (Bottom) The densitometric values of labeled Na,K-ATPase α-subunit bands were expressed as a percentage of the optical density value of cells that were incubated in isotonic medium (100%). Results are means ± SEM from four separate experiments. *P < 0.05 versus control 300 mOsm/L values. (D) Confluent mpkCCD_{c14} cells that were preincubated in the presence of isotonic (300 mOsm/L) NaCl incubation solution were incubated in either an isotonic or a hypotonic (200 mOsm/L) NaCl solution for 1 h at 37°C. The α-transferrin receptor (top), E-cadherin (middle), and Na,K-ATPase α-subunit (bottom) were detected by Western blotting performed on the same membrane after biotinylation and streptavidin precipitation of cell surface proteins.

These results demonstrate that extracellular hypotonicity specifically induces the recruitment of Na,K-ATPase and functionally linked E-cadherin to the cell surface. Moreover, this effect critically depends on apical Na⁺ influx mediated by ENaC.

Discussion

The present study shows that extracellular hypotonicity and amphotericin B induce the recruitment of Na,K-ATPase units to the cell surface of renal CD principal cells independent of cell volume variation *via* increased Na⁺ influx, leading to the proteasomal-dependent activation of PKA. Renal CD cells are exposed to physiologic variations in luminal fluid and/or interstitial osmolality, which influence cell volume (22) and trigger adaptive processes aimed at restoring initial cell volume (23,24). Indeed, renal epithelial cells undergo cell swelling or cell shrinkage in response to extracellular hypotonicity or hypertonicity, respectively (25–27). That Na,K-ATPase provides the driving force for vectorial ion transport and plays a key role in the maintenance of a constant composition of the intracellular milieu and cell volume (28) led us to analyze the existing relationship between cell volume variation and Na,K-ATPase cell surface expression, a major determinant of Na,K-ATPase activity in CD principal cells (3). In agreement with a previous study on isolated mouse CCD (9), the present findings demonstrate that hypotonicity, which induced cell swelling, is associated with Na,K-ATPase recruitment to the plasma membrane but that hypertonicity, which induced cell shrinkage, does not alter Na,K-ATPase cell surface expression. Modification of the intracellular concentration or activity of solutes may also lead to variations of cell volume (26,27,29), particularly in secreting or absorptive epithelial cells. For example, apical Na⁺-alanine and Na⁺-glucose co-transporter activation induced a sustained increase in cell volume in isolated rabbit proximal tubules (26). Likewise, inhibition of active Na⁺ extrusion by ouabain, which specifically inhibits the Na,K-ATPase, increases cell volume in CCD (29). It is interesting that the Na⁺ ionophore amphotericin B induces a similar increase in Na,K-ATPase cell surface expression under isotonic conditions, *i.e.*, in the absence of cell volume variation, as well as under hypotonic or hypertonic conditions associated with cell shrinkage or cell swelling, respectively. These results strongly suggest that the expression levels of cell surface Na,K-ATPase are not directly controlled by cell volume variation in renal CD principal cells (Table 2).

Previous studies performed in renal A6 cells have shown that a short hypotonic challenge stimulates both Na,K-ATPase activity (7) and apical Na⁺ entry *via* an increase in the number of conducting ENaC (4–6). Results of the present study confirm that extracellular hypotonicity stimulates apical Na⁺ influx through ENaC (Figure 6) and show that elimination of the apical Na⁺ entry through ENaC either by addition of 10⁻⁶ M amiloride to the apical side of mpkCCD_{c14} cells grown on filters or by incubation of cells in a nominally Na⁺-free solution fully prevented the increase in Na,K-ATPase cell surface expression induced by extracellular hypoosmolality (Figures 7 and 8). Moreover, exposure of mpkCCD_{c14} cells to hypotonic

sucrose medium that contained or not 40 mM Na⁺ induced a similar increase in cell volume. These observations therefore suggest that the increase in [Na_i]⁺ consecutive to the stimulation of ENaC-dependent Na⁺ entry and not cell swelling triggers the increase in Na,K-ATPase cell surface expression in response to a short-term hypotonic challenge in mpkCCD_{c14} cells. However, our results do not rule out the possibility that cell swelling triggers ENaC activation in response to extracellular hypotonicity. This finding is in agreement with the previously described intracellular Na⁺ concentration-dependent recruitment of Na,K-ATPase in mpkCCD_{c14} cells (19). Altogether, our observations suggest that CD principal cells exhibit an intracellular Na⁺ sensing mechanism that triggers a signaling cascade that leads to Na,K-ATPase recruitment from an inactive intracellular pool to the basolateral plasma membrane, as described previously in response to cAMP (12), aldosterone (13), activation of G protein-coupled receptors (30), or increased [Na_i]⁺ (19,31).

We have previously shown that a rise in [Na_i]⁺ induced by Na⁺ ionophores leads to proteasomal-dependent PKA activation and subsequent increased expression of cell surface Na,K-ATPase in mpkCCD_{c14} cells (19). Here we show that both hypotonicity and the Na⁺ ionophore amphotericin B stimulate to the same extent and in a nonadditive manner the recruitment of cell surface Na,K-ATPase (Figure 2). Furthermore, Na,K-ATPase cell surface recruitment could be prevented after pharmacologic inhibition of either PKA or proteasomal activity. Altogether, these results strongly suggest that extracellular hypotonicity and amphotericin B share the same signaling pathway. The Na⁺-induced activation of PKA does not require cAMP (19), thus excluding the classical pathway involving dissociation of the PKA holoenzyme induced by cAMP binding to regulatory subunits, which consequently alleviates autoinhibitory contacts and releases the active PKA catalytic subunit (PKAc) (32,33). Ferraris *et al.* (34) recently showed that, in response to extracellular hypertonicity, PKA can be activated independent of cAMP after the release of free active PKAc as a result of the dissociation of a protein complex that comprises a discrete pool of PKAc associated with the tonicity-responsive enhancer binding protein. However, such a mechanism of PKA activation caused by increased cellular Na⁺ influx seems to be very unlikely because extracellular hypertonicity, a hallmark of tonicity-responsive enhancer binding protein activation (34), does not alter Na,K-ATPase cell surface expression (Figure 1). Recently, Zhong *et al.* (35) demonstrated that the transcriptional activity of NF-κB is regulated by the IκB-associated PKAc subunit activation through a cAMP-independent pathway. Inducers such as LPS and IL-1 initiate a cytoplasmic signaling cascade that ultimately leads to the proteasomal degradation of IκB, nuclear translocation of NF-κB, and transcriptional activation of proinflammatory response genes. It is interesting that among the cascade of events that control this complex process, the proteasomal degradation of IκB was shown to result in cAMP-independent activation of PKAc, which forms a multimeric PKAc/IκBα/NF-κB p65 complex under inactive state (35,36). The proteasomal dependence of both amphotericin B-induced (19) and extracellular

hypotonicity-induced cell surface recruitment of Na,K-ATPase (Figure 5) is compatible with the mechanism reported above and requires further investigation. It remains to be determined whether PKA directly induces Na,K-ATPase redistribution, *e.g.*, through phosphorylation of the Na,K-ATPase α -subunit (37–39), or requires further downstream signaling intermediate(s).

In conclusion, our results strongly suggest that Na⁺ ionophores and extracellular hypotonicity induce recruitment of Na,K-ATPase units to the plasma membrane after similar cAMP-independent activation of PKA, most likely through the release of active PKAc from a protein complex after proteasomal degradation of a regulatory protein in response to increased [Na⁺]_i. This process is not directly dependent of cell volume variation and most likely relies on the activation of an intracellular Na⁺-sensing pathway.

Acknowledgments

This work was supported in part by Swiss National Foundation Grant 31-67878.02 and a Carlos and Elsie deReuter Foundation grant to E.F.

We thank Fabio Carrozzino and Michelangelo Foti (Department of Morphology, University of Geneva, Geneva, Switzerland) for helpful discussion and technical skills.

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