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Involvement of Protein Tyrosine Kinase in Osmoregulation of Na^+ Transport and Membrane Capacitance in Renal A6 Cells

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Abstract. Renal A6 cells have been reported in which hyposmolality stimulates Na^+ transport by increasing the number of conducting amiloride-sensitive 4-pS Na^+ channels at the apical membrane. To study a possible role of protein tyrosine kinase (PTK) in the hyposmolality-induced signaling, we investigated effects of PTK inhibitors on the hyposmolality-induced Na^+ transport in A6 cells. Tyrphostin A23 (a PTK inhibitor) blocked the stimulatory action of hyposmolality on a number of the conducting Na^+ channels. Tyrphostin A23 also abolished macroscopic Na^+ currents (amiloride-sensitive short-circuit current, I_{Na}) by decreasing the elevating rate of the hyposmolality-increased I_{Na}. Genistein (another type of PTK inhibitor) also showed an effect similar to tyrphostin A23. Brefeldin A (BFA), which is an inhibitor of intracellular translocation of protein, blocked the action of hyposmolality on I_{Na} by diminishing the elevating rate of the hyposmolality-increased I_{Na}, mimicking the inhibitory action of PTK inhibitor. Further, hyposmolality increased the activity of PTK. These observations suggest that hyposmolality would stimulate Na^+ transport by translocating the Na^+ channel protein (or regulatory protein) to the apical membrane via a PTK-dependent pathway. Further, hyposmolality also caused an increase in the plasma (apical) membrane capacitance, which was remarkably blocked by treatment with tyrphostin A23 or BFA. These observations also suggest that a PTK-dependent pathway would be involved in the hyposmolality-stimulated membrane fusion in A6 cells.

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Key words: Amiloride — Na^+ channel — Brefeldin A — ENaC — Genistein — Tyrphostin A23 — Hyposmolality

Introduction

A wide variety of cells can adapt themselves to new environments and can respond to environmental changes, including osmotic stress, in multiple ways. When cells are exposed to high osmolality, cells recover their cell volume by taking solutes up from the extracellular site (volume regulatory increase) after the first shrinkage caused by water loss, since hyperosmotic stress activates ion channels and ion transporters to accumulate solutes for diminishing osmotic gradient across the plasma membrane. Conversely, when cells are exposed to low osmolality, water influx due to osmotic gradient across the plasma membrane rapidly causes cell swelling. After the initial swelling, cells undergo slower, compensatory shrinkage through loss of intracellular solutes via a process known as regulatory volume decrease (RVD). In addition, the hyposmolality-induced cell swelling causes a change in membrane tension. The signal transduction mechanisms activated by a change in extracellular osmolality, in turn, produce adaptive responses such as activation of ion transport systems [5, 38, 46] or gene transcription, especially ion channels and ion transporters [6, 24, 57].

Hyposmotic stress activates various signaling pathways which increase the cytosolic Ca^{2+} concentration [51, 61], cyclic AMP [58], inositol 1,4,5-triphosphate and arachidonic acid metabolites [54]. Hyposmotic stress also influences a variety of ion channels and ion

transporters to decrease cell volume by loss of solutes, such as volume-sensitive Cl^- and K^+ channels [3, 59], KCl cotransporter [47], and the Na^+, K^+-ATPase [44]. In addition, hyposmotic stress increases protein tyrosine phosphorylation in human intestine cells [55] and in human neutrophils [15]. These reports suggest that hyposmotic stress causes activation of protein tyrosine kinase (PTK). A more recent study in cardiac myocytes [52] provides evidence that hyposmotic stress immediately activates PTK and is mimicked by chlorpromazine which is known to cause membrane deformation. This report [52] suggests that PTK activation may require a change in membrane tension caused by hyposmotic stress and that the activated PTK may play an essential role in response to environmental changes.

The renal epithelium is one of the tissues that is routinely exposed, under physiological conditions, to variable extracellular osmolality. A6 cells derived from the kidney of *Xenopus laeves* are used for studies on epithelial Na^+ transport [9, 29] which is regulated by various factors such as aldosterone [1, 19], antidiuretic hormone (ADH) [28] and extracellular osmolality [43, 60]. Apical uptake of Na^+ occurs through an amiloride-blockable highly Na^+-selective channel with a small single channel conductance (4 pS) [29] that is the predominant Na^+-permeable channel [27]. The rate-limiting step of transepithelial Na^+ transport in A6 cells is, in general, the Na^+ entry step through the amiloride-blockable Na^+ channel in the apical membrane [26, 27]. Therefore, the regulation of the number and/or activity of Na^+ channels in the apical membrane is required for stimulation of the transepithelial Na^+ transport. Hyposmotic stress stimulates Na^+ transport through amiloride-blockable Na^+ channels in the apical membrane [43, 60]. However, the signal transduction pathways involved in regulation of the Na^+ channel including channel density and individual channel activity are still unknown. This study focused on the investigation of the signal transduction mechanisms responsible for the activation of Na^+ transport by hyposmolality. We demonstrate here that hyposmotic stress stimulates Na^+ transport by increasing the number and activity of the amiloride-blockable 4-pS Na^+ channel in the apical membrane of A6 cells via activation of PTK.

Materials and Methods

Cell Culture

A6 cells were purchased from American Type Culture Collection (Rockville, MD) at passage 68. Passages 76–84 were used for experiments. No differences were discernible between cells from different passages. Cells were maintained in plastic tissue culture flasks at 27°C in a humidified incubator with 2% CO_2 in air in a culture medium, which contained 75 % (volume / volume (v/v)) NCTC-109 medium (GIBCO, Grand Island, NY, 15 % (v/v) distilled water and 10% (v/v) fetal bovine serum (GIBCO) [40].

Solutions

The osmolality of culture medium used in the present study was 255 mOsm/kg H_2O. An isosmotic solution used in the present study, which had the same osmolality as the culture medium (255 mOsm/kg H_2O), contained the following ion concentrations (in mM); 95 NaCl, 3.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 glucose, 10 N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES), and 25 $NaHCO_3$. Hyposmotic solutions used in the present study contained the same ion concentrations as the isosmotic solution except for the NaCl concentration; i.e., a hyposmotic solution with 135 mOsm/kg H_2O contained 30 mM NaCl, and a hyposmotic solution with 200 mOsm/kg H_2O contained 65 mM NaCl. The pH of solutions used in the present study was adjusted to 7.4 by NaOH before addition of $NaHCO_3$. Bathing solutions were stirred with 5% CO_2/21 % O_2/74 % N_2. Unless otherwise stated, a hyposmotic solution containing 30 mM NaCl with 135 mOsm/kg H_2O was used for application of hyposmolality. Dimethyl sulfoxide (DMSO) was used as solvent for tyrphostin A23, genistein, brefeldin A and amiloride. DMSO alone used in the preset study had no effects on currents.

Short-Circuit Current (I_{sc})

Monolayers of cells subcultured on Tissue culture-treated Transwell filter cups (COSTAR® Transwell™, Tissue culture-treated Transwell; Costar Corporation, Cambridge, MA) for 10–14 days were transferred to a modified Ussing chamber (Jim's Instrument, Iowa City, IA) designed to hold the filter cup [41, 42]. Transepithelial potential (PD) was continuously measured by a high-impedance millivoltmeter that could function as a voltage clamp with automatic fluid resistance compensation (VCC-600, Physiologic Instrument, San Diego, CA) with a pair of calomel electrodes that were immersed in a saturated KCl solution and bridged to the modified Ussing chamber by a pair of polyethylene tubes filled with a solution of 2% (weight/volume (w/v)) agarose in a 2M KCl solution [22, 40]. I_{sc} was measured by the amplifier, VCC-600, with a pair of silver-silver chloride electrodes that were immersed in 2 M NaCl solution and bridged to the modified Ussing chamber by a pair of polyethylene tubes filled with a solution of 2% (w/v) agarose in 2 M NaCl solution [41, 42]. When the I_{sc} was measured, the PD was clamped to 0 mV for 1 sec by the amplifier. Under a steady condition, the I_{sc} was stable and did not change even if the transepithelial voltage was clamped to 0 mV for up to 1 min. In a nonsteady state, the value of I_{sc} measured at 1 sec after clamping the PD to 0 mV is shown as I_{sc} in the present study. A positive current represents a net flow of cation from the apical to the basolateral solution. The experiments were performed at 24–25°C.

Single Channel Current Recording

Cells were subcultured on translucent porous Nunc filter inserts (Nunc Tissue Culture Inserts; Nunc, Roskilde, Denmark) for 10–14 days before applying the single channel recording technique [29, 35]. Standard patch-clamp techniques were applied to a confluent monolayer of A6 cells [21, 31–33]. Patch pipettes were made from LG 16 glass (Dagan Corporation, Minneapolis, MN) and fired-polished to produce tip diameters of about 0.5 μm. The patch pipette was applied from the apical side. Then, we made a GΩ seal (>100 GΩ) on the apical membrane of cells. Single channel currents were obtained with an Axopatch

1D patch-clamp amplifier (Axon Instruments, Foster City, CA). Current signals were recorded on a digital video recorder (HF860D, Sony, Tokyo, Japan) with pulse-code modulation (1-DR-390, NeuroData Instruments Corporation, New York, NY). Current signals were digitized at a sampling rate of 5,000 Hz, and analyzed with a continuous-data acquisition program. A 100-Hz low-pass filter using a software Gaussian filter was used to present the actual traces. The open probability was measured at no applied potential in cell-attached patches (i.e., resting membrane potential) [28, 32, 34]. The experiments were performed at 24–25°C.

Membrane Capacitance

Cells were subcultured on porous Millicell HA filters (Millipore, Bedford, MA), for 10–14 days before measuring membrane capacitance (C_T). C_T was measured with 5 sine waves which were repetitively imposed to the epithelium. The frequencies used for these measurements were 2.0, 2.7, 4.1, 5.4 and 8.2 kHz. In this frequency range, the AC characteristics of the epithelium can be described by a lumped RC network consisting of the parallel arrangement of a capacitor (C_T) and a resistor (R_T) in series with a resistance (*Rs*) attributed to the bathing solution between the voltage electrodes. *Rs* was determined as the extrapolated intercept of the impedance function with the real axis in the Nyquist diagram. In the frequency range above 2 kHz C_T equals the equivalent capacitance of the series arrangement of the apical and basolateral membranes. Since the basolateral C_T in A6 epithelia is about 10–12 larger than that of the apical membrane [17], C_T would be mainly determined by the apical C_T. Within this configuration, increases of C_T in the order of 15% as found in this study could only be attributed to the apical border. Within the frequency range used (2–8.2 kHz) C_T did not noticeably change with frequency. This observation validates the use of C_T as index for membrane area. The experiments were performed at 24–25°C.

Western Blotting

Cells were subcultured on translucent porous Nunc filter inserts (Nunc Tissue Culture Inserts, Nunc, Roskilde, Denmark) for 10–14 days before Western blot experiments. Cells with and without hyposmotic treatment were lysed by Lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 100 mM NaF, 10 mM pyrophosphate, 200 μM Na-orthovanadate, 250 μg/ml leupeptin, 0.1 mM phenyl-methylsulfonyl fluoride, 100 kallikrein inactivator units/ml aprotinin, pH 7.4) on ice. Cells were homogenized by sonication and centrifuged at 12,000 × *g* for 10 min at 4°C to remove insoluble debris. The cell lysate containing 25 μg protein was boiled in SDS sample buffer (60 mM Tris-HCI, 2% (w/v) SDS, 5% (v/v) glycerol, pH 6.8) and then subjected to 10% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to nitrocellulose membranes. Nonspecific binding was blocked by incubation in 5% (w/v) bovine serum albumin for 60 min. Membranes were immunoblotted with a monoclonal anti-phosphotyrosine antibody, PY99 (Santa Cruz Biotechnology, Santa Cruz, CA). After overnight incubation at 4°C, the membrane was incubated for 60 min at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG. After washing, blots were developed with an enhanced chemiluminescence (ECL) detection kit from Amersham (Oakville, Ontario, Canada). The intensity of band was quantified with an Imaging Densitomer (GS-690, Bio-Rad Laboratories, Hercules, CA).

PTK Activity

Cells were subcultured on translucent porous Nunc filter inserts for 10–14 days before measuring PTK activity. To determine PTK activity, cells with and without hyposmotic treatment were lysed by radioimmune precipitation assay (RIPA) buffer (1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 1% (w/v) SDS, 158 mM NaCl, 10 mM Tris, 1 mM EGTA, 250 μg/ml leupeptin, 0.1 mM phenyl-methylsulfonyl fluoride, 1 mM benzamidine, 10 mM Na_3VO_4, 100 kallikrein inactivator units/ml aprotinin, pH 7.2). Cells were homogenized by sonication and centrifuged at 12,000 × *g* for 10 min at 4°C to remove insoluble debris. Supernatants were stored as aliquots at −80°C. The PTK activity was measured with an ELISA based tyrosine kinase assay kit from Pierce (Rockford, IL). Briefly, a biotinylated tyrosine kinase peptide substrate, synthetic human gastrin-17, was added to 96-well NeutrAvidin plate and incubated for 30 min at 37°C. An aliquot of the supernatant of cell lysates (10 μl) was incubated with reaction buffer containing ATP and Mg^{2+}, incubated for 45 min at 30°C. An HRP-labeled anti-phosphotyrosine (PY20, 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) was added to the plate, followed by 1 hr incubation at 37°C. HRP substrate, 1-Step™ Turbo TMB solution was added for approximately 5–15 min for coloring reaction, which was stopped by adding equal volume of 1 N H_2SO_4. The plate was washed three times with 250 μl of TBS. The optical density read at 650 nm was measured and calculated against a standard curve which was made by using tyrosine phosphorylated synthetic human gastin-17. The final results are expressed as tyrosine phosphorylation of peptide substrate (μg/min) per mg protein in cell lysates.

Materials

Amiloride and brefeldin A were purchased from SIGMA (St. Louis, MO). Tyrphostin A23 and genistein were obtained from CALBIOCHEM (San Diego, CA).

Data Presentation

All data shown in the present study are represented as means ± standard errors (SE). Where SE bars are not visible, they are smaller than the symbol. The Student's *t*-test, ANOVA and Ducan's multiple range comparison test were used for statistical analysis as appropriate and the P value < 0.05 was considered significant.

Results

Effects of Hyposmolality on the Number and Open Probability (*Po*) of Amiloride-Blockable Na^+ Channels and an Inhibitory Action of Tyrphostin A23

We assessed the effect of hyposmolality on the number of conducting Na^+ channels in the apical membrane by recording single N^+ channel currents. We could detect approximately one Na^+ channel with a single channel conductance of 4 pS per patch membrane (Figs. 1*A*, 2*A* and *B*) in an isosmotic solution (bathing as well as pipette solutions), which was highly Na^+ selective and sen-

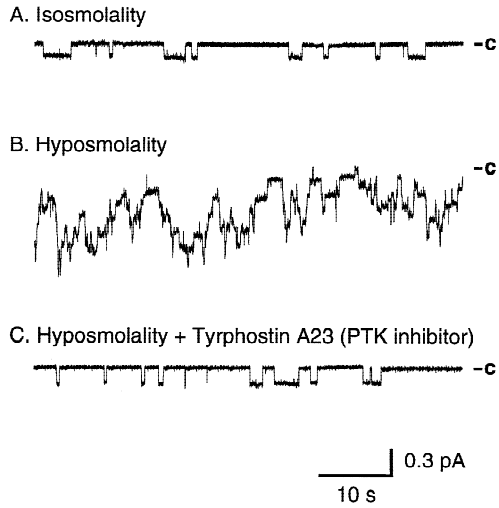

Fig. 1. Effects of hyposmotic stress on single Na^+ channel currents in the apical membrane. (*A*) Isosmotic solution (255 mOsm/kg H_2O; both bathing and pipette solutions were the isosmotic solution). (*B*) The single channel current was recorded at 120 min after exposure of cells to a hyposmotic solution (135 mOsm/kg H_2O). Both bathing and pipette solutions were hyposmotic. (*C*) Hyposmotic solution (135 mOsm/kg H_2O) with 100 μM tyrphostin A23. Cells were pretreated with 100 μM tyrphostin A23 (bilateral application) for 60 min in an isosmotic solution and subsequently incubated in a hyposmotic solution (135 mOsm/kg H_2O) containing 100 μM tyrphostin A23 for the both apical and basolateral solutions. The single channel current was recorded at 120 min after exposure of cells to a hyposmotic solution (135 mOsm/kg H_2O) containing 100 μM tyrphostin A23 for the both apical and basolateral solutions. Both bathing and pipette solutions were hyposmotic. The closed level of single channel current within patch is marked with a dash line and "c". Inward current is downward. The single channel currents were recorded in cell-attached patches at no applied pipette potential (resting apical membrane potential).

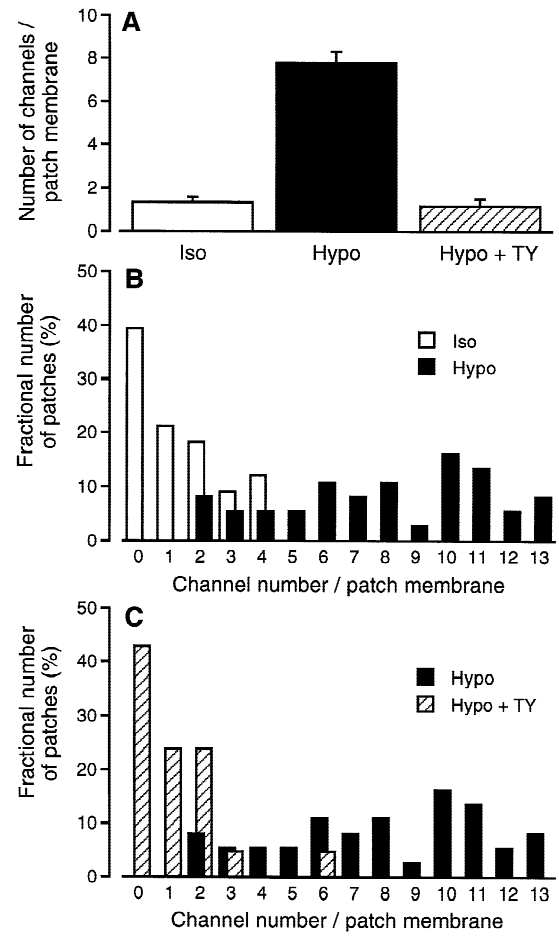

Fig. 2. Hyposmolality-induced increase in the number of Na^+ channels in the apical membrane. (*A*) The number of conducting Na^+ channels per patch membrane. (*B*) The histogram of conducting Na^+ channels per patch membrane obtained from cells incubated in isosmotic and hyposmotic solution. (*C*) The histogram of conducting Na^+ channels per patch membrane obtained from cells treated with and without tyrphostin A23 in the hyposmotic solution. **Iso;** Isosmotic solution (255 mOsm/kg H_2O; both bathing and pipette solutions were isosmotic). $n = 33$. **Hypo:** Single channel currents were recorded at 120 min after exposure of cells to a hyposmotic solution (135 mOsm/kg H_2O). Both bathing and pipette solutions were hyposmotic. $n = 37$. **Hypo + TY:** Hyposmotic solution (135 mOsm/kg H_2O) with 100 μM tyrphostin A23. Cells were pretreated with 100 μM tyrphostin A23 (bilateral application) for 60 min in an isosmotic solution of 255 mOsm/kg H_2O and subsequently incubated in a hyposmotic solution (135 mOsm/kg H_2O) containing 100 μM tyrphostin A23 for the both apical and basolateral solutions. The single channel current was recorded at 120 min after incubation of cells in a hyposmotic solution (135 mOsm/kg H_2O) containing 100 μM tyrphostin A23 for the both apical and basolateral solutions. Both bathing and pipette solutions were hyposmotic. $n = 21$. Exposure to a hyposmotic solution significantly increased the number of conducting Na^+ channels per patch membrane, and this effect was blocked by tyrphostin A23.

sitive to amiloride as reported previously [29]. Unless otherwise stated, a hyposmotic solution with 135 mOsm/kg H_2O was used for application of hyposmolality. The number of Na^+ channels per patch membrane dramatically increased about 6-fold after exposure to a hyposmotic solution about 120 min (Figs. 1*B*, 2*A* and *B*). We also analyzed the channel activity (open probability) of the channel under isosmotic and hyposmotic conditions. As shown in Fig. 3, hyposmolality increased the P_o of the channel about 1.5-fold. These observations suggest that hyposmolality increased the number and P_o of conducting Na^+ channels in the apical membrane.

We next examined whether PTK is involved in the hyposmolality-increased number and P_o of amiloride-blockable Na^+ channels. To assess the effect of tyrphostin A23, a PTK inhibitor, on the increase in number and P_o of the Na^+ channel under a hyposmotic condition, we made cell-attached patches on cells that were pretreated with 100 μM tyrphostin A23 for 60 min prior to hyposmotic exposure and subsequently exposed to a hyposmotic solution containing 100 μM tyrphostin A23. Under this condition (tyrphostin A23 treatment) the number of Na^+ channels per patch membrane (Figs. 1*C*, 2*A* and *C*) was significantly smaller than that without tyrphostin A23 treatment (Figs. 1*B*, 2*A* and *B*), and was almost

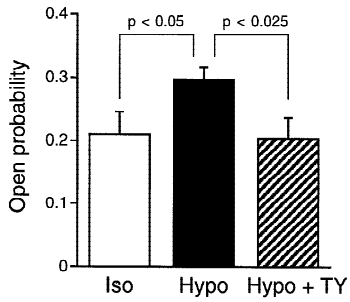

Fig. 3. Hyposmolality-induced increase in the open probability (P_o) of Na^+ channels in the apical membrane. **Iso:** Isosmotic solution (255 mOsm/kg H_2O; both bathing and pipette solutions were isosmotic). $n = 9$. **Hypo:** Single channel currents were recorded at 120 min after exposure of cells to a hyposmotic solution (135 mOsm/kg H_2O). Both bathing and pipette solutions were hyposmotic. $n = 11$. **Hypo + TY:** Hyposmotic solution (135 mOsm/kg H_2O) with 100 μM tyrphostin A23. Cells were pretreated with 100 μM tyrphostin A23 (bilateral application) for 60 min in an isosmotic solution of 255 mOsm/kg H_2O and subsequently incubated in a hyposmotic solution (135 mOsm/kg H_2O) containing 100 μM tyrphostin A23 for the both apical and basolateral solutions. The single channel current was recorded at 120 min after incubation of cells in a hyposmotic solution (135 mOsm/kg H_2O) containing 100 μM tyrphostin A23 for the both apical and basolateral solutions. Both bathing and pipette solutions were hyposmotic. $n = 9$. Exposure to a hyposmotic solution significantly increased the open probability of the channel, and this effect was blocked by tyrphostin A23. We did not use the patch that had no channel activity.

identical to that under an isosmotic condition (Figs. 1*A* and 2). The increase in P_o caused by hyposmolality was also blocked by tyrphostin A23 (Figs. 1*B* and *C*, and 3).

Effects of Hyposmolality on Short-Circuit Current (I_{sc})

We also examined the effects of osmolality of the bathing solution on macroscopic Na^+ current which was detected as amiloride-sensitive short-circuit current (I_{Na}) by exposure to an isosmotic (255 mOsm/kg H_2O) or a hyposmotic solution (135 mOsm/kg H_2O). The I_{sc} gradually increased with time after exposure of A6 cells to a hyposmotic solution with 135 mOsm/kg H_2O. Most of the increased I_{sc} was sensitive to 10 μM amiloride (Fig. 4*A*, open circles). The hyposmolality-induced increase in I_{sc} reached a stable, sustained level over 90 min after exposure of the cell to a hyposmotic solution. However, in an isosmotic solution whose osmolality was identical to the culture medium, no increase in I_{Na} was observed (Fig. 4*A*, closed circles). Thus, the I_{Na} increased as osmolality of the bathing solution decreased (Fig. 4*B*).

Since we measured Na^+ transport as $I_{Na,}$ we next examined whether solution osmolality affects amiloride-sensitivity of I_{sc}. Figure 5 shows dose-dependent effects

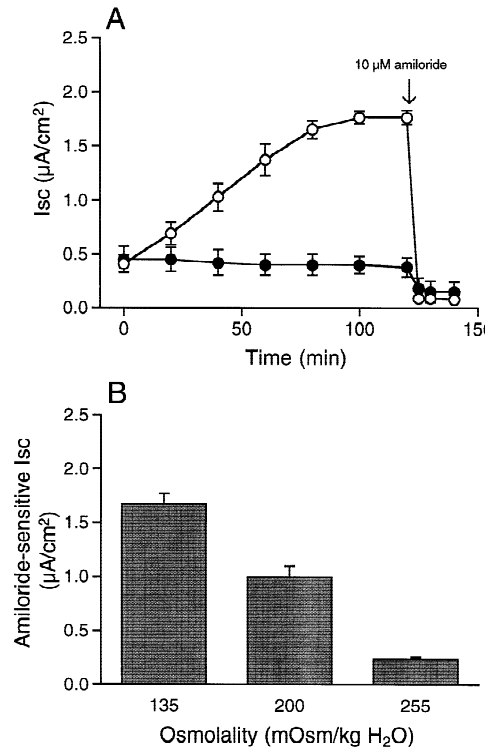

Fig. 4. Effect of extracellular osmolality on I_{Na}. (*A*) Time-dependent changes in I_{sc} under isosmotic (255 mOsm/kg H_2O, closed circles, mean ± SE, the number of experiments (n) = 6) and hyposmotic (135 mOsm/kg H_2O, open circles, mean ± SE, $n = 6$) conditions. (*B*) I_{Na} under isosmotic and hyposmotic conditions 120 min after exposure to the solutions.

of amiloride on the I_{sc} in isosmotic and hyposmotic solutions. Similar to a previous report [60], the sensitivity of I_{sc} in a hyposmotic solution with 135 mOsm/kg H_2O to amiloride was similar to that in an isosmotic solution with 255 mOsm/kg H_2O. In each case, the concentration showing the half maximum inhibition (IC_{50}) of amiloride was about 0.1 μM. The IC_{50} obtained at a macroscopic current level (I_{Na}) in the present study is identical to the IC_{50} obtained at a single-channel current level, an amiloride-sensitive 4-pS highly Na^+-selective channel [13], which is the predominant amiloride-sensitive Na^+-permeable channel contributing to transepithelial Na^+ transport in A6 cells [28, 29]. These results suggest that a decrease in solution osmolality has no effects on the amiloride-sensitivity of the $I_{sc,}$ and that an amiloride-sensitive 4-pS Na^+ channel would account for the hyposmolality-induced I_{Na} as well as the I_{Na} under an isosmotic condition.

Effects of PTK Inhibitors on Hyposmolality-Induced I_{sc}

In several types of cells, osmotic shock (or stress) activates a variety of signaling pathways which result in

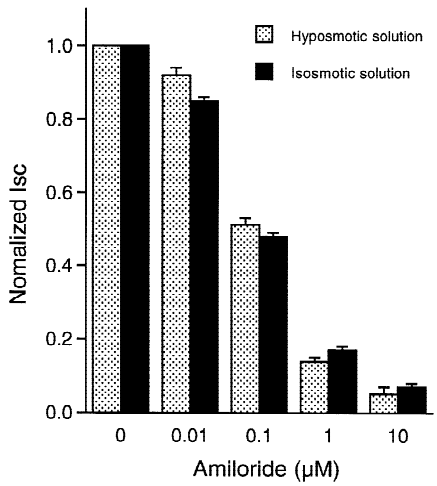

Fig. 5. Dose-dependent effects of amiloride on hyposmolality-induced I_{sc}. After incubation in a hyposmotic solution (135 mOsm/kg H_2O, dotted columns) or an isosmotic solution (255 mOsm/kg H_2O, solid columns) for 120 min, amiloride (10 nM − 10 μM) was applied to the apical bathing solution ($n = 4$).

specific cellular responses. Particularly, phosphorylation of tyrosine residues is one of the first events in a variety of signaling pathways [55].

To study a possible role of PTK in the hyposmolality-stimulated Na^+ transport, we examined effects of PTK inhibitors on the hyposmolality-induced increase in I_{Na}. Tyrphostin A23 (a PTK inhibitor, 100 μM; pretreatment for 60 min before exposure of cells to hyposmolality and subsequent presence in a hyposmotic solution) significantly diminished the action of hyposmolality on I_{sc} (Fig. 6*A*). We also applied tyrphostin A23 (10–100 μM) to bilateral sites after the hyposmolality-induced I_{sc} reached a sustained phase. The post-treatment with 100 μM tyrphostin A23 also rapidly and significantly reduced the hyposmolality-induced I_{sc} (Fig. 6*B*). Tyrphostin A23 (100 μM) almost completely inhibited I_{sc} 60 min after its application (Fig. 6*B*). Figure 6*C* and *D* illustrate the dose-dependent inhibition of the I_{Na} by tyrphostin A23. Another PTK inhibitor, genistein, also showed an inhibitory effect on the hyposmolality-induced I_{Na} (Fig. 7). Pre- and post-treatments with 200 μM genistein had markedly inhibitory effects on the I_{sc} (Figs. 7*A* and *B*). Dose-dependence in genistein is illustrated in Figs. 7*C* and *D*. These results suggest a possible role of PTK in the signal transduction pathway involved in the hyposmolality-induced increase of I_{Na}.

To further analyze the effects of PTK inhibitors, tyrphostin A23 and genistein on the hyposmolality-induced I_{sc}, we normalized the I_{sc} using the same data as those shown in Figs. 6*A* and 7*A*, in which cells were pretreated with tyrphostin A23 and genistein, respectively. Tyrphostin A23 and genistein diminished the increasing rate of the I_{sc} induced by hyposmolality. In this plot (Figs. 8*A* and *B*), the effect of tyrphostin A23 and genistein on the basal I_{sc} is normalized. Therefore, the diminution of the increasing rate of I_{sc} by tyrphostin A23 and genistein suggest that tyrphostin A23 or genistein really diminish the stimulatory action of hyposmolality on I_{sc}, but the diminution of I_{sc} by tyrphostin A23 or genistein is not only due to the inhibitory action on the basal I_{sc} (not due to proportional inhibition to the basal I_{sc}).

Effects of Hyposmolality on Tyrosine Phosphorylation

Since PTK inhibitors significantly diminished the hyposmolality-induced increased in I_{sc}, we examined whether hyposmotic stress induces protein tyrosine phosphorylation in A6 cells. Using immunoblotting with anti-phosphotyrosine antibody, we found that the hyposmotic stress caused marked increases in phosphotyrosine content of multiple proteins (Fig. 9*A*). These proteins had molecular sizes of ~40, ~60, ~80, ~110, ~130 and ~200 kDa. Its initial increase was already observed within 1 min after exposure to a hyposmotic solution reaching a peak 5 min after hyposmotic exposure. The increased level of phosphotyrosine was maintained over 90 min (Fig. 9*A*). The time course of phosphorylation of tyrosine shown here was very different from that of the I_{Na}. Especially, tyrosine phosphorylation of protein(s) ranging around 60 kDa obviously increased more than 3-fold about 5 min after exposure to a hyposmotic solution (Fig. 9*A* and *B*). On the other hand, the I_{sc} did not reach a peak value, but increased gradually with time over 60–90 min after exposure of cells to a hyposmotic solution (Fig. 4*A*) (*see* argument about the different time courses in Discussion in detail).

Effects of Hyposmolality on Activity of PTK

This result on phosphotyrosine induced by hyposmolality is consistent with our previous observation [44], and in the present study we present a longer time course of changes in the amount of phosphotyrosine after exposure of A6 cells to a hyposmotic solution. This observation suggests that the increase in tyrosine phosphorylation would be due to an increase in PTK activity. However, it is still unknown whether hyposmotic stress really increases PTK activity. To confirm whether hyposmotic stress activates PTK, we measured PTK activity in the cell lysates by using a synthetic PTK substrate. PTK activity significantly increased about 2-fold by hyposmotic stress (Fig. 10).

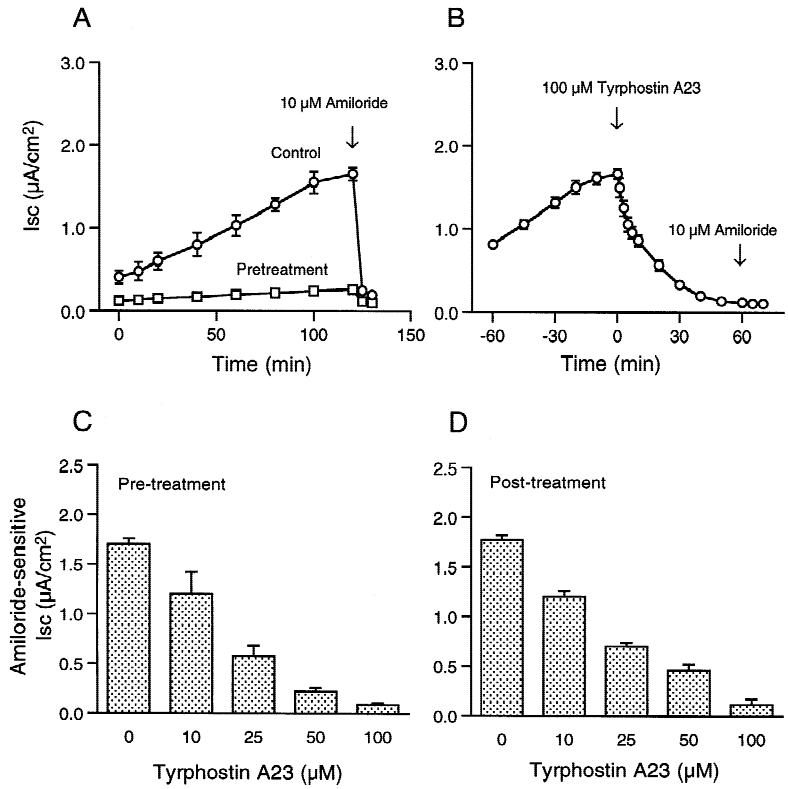

Fig. 6. Effects of tyrphostin A23 on hyposmolality-induced I_{Na}. (*A*) Effect of pretreatment with tyrphostin A23 on hyposmolality-induced I_{sc}. The I_{sc} was measured in a hyposmotic solution of 135 mOsm/kg H_2O without (circles) and with (squares) 100 μM tyrphostin A23 (bilateral pretreatment for 60 min and presence during the period for measurement of I_{sc}). $n = 4$–5. (*B*) Effect of post-treatment with tyrphostin A23 on hyposmolality-induced I_{sc}. After the I_{sc} reached a sustained phase in a hyposmotic solution of 135 mOsm/kg H_2O, 100 μM tyrphostin A23 was added to the bilateral sites. $n = 4$. (*C*) Dose-dependent effects of tyrphostin A23 pretreatment on the hyposmolality-induced I_{Na} ($n = 4$–5). The protocol of the experiment was the same as that shown in Fig. 6*A*. (*D*) Dose-dependent effects of tyrphostin A23 post-treatment on the hyposmolality-induced I_{Na} ($n = 4$–6). The protocol of the experiment was the same as that shown in Fig. 6*B*.

Effects of Brefeldin A (BFA) on I_{sc}

To understand the mechanism of the hyposmolality-induced stimulation of Na^+ transport, we assessed the effect of brefeldin A (BFA), a blocker of translocation of proteins from endoplasmic reticulum (ER) to the Golgi apparatus [37], on the hyposmolality-induced I_{Na}. BFA (5 μg/ml; pretreatment for 60 min before exposure of cells to hyposmolality and subsequent presence in a hyposmotic solution) significantly attenuated the hyposmolality-induced I_{sc} (Fig. 11*A*). Pretreatment with BFA for 60 min diminished the basal I_{sc} (*see* the I_{sc} at time 0 in Fig. 11*A*). Therefore, the inhibitory effect of BFA on the hyposmolality-induced I_{sc} might be due to the inhibitory action on the basal I_{sc} (proportional inhibition), but not mediated through its inhibition of the signaling of hyposmolality on the I_{sc}. To clarify this point, we normalized the action of hyposmolality on the I_{sc} in the presence and absence (control) of BFA. As shown in Fig. 11*B*, BFA diminished the increasing rate of I_{sc} induced by hyposmolality, indicating that BFA diminishes the stimulatory action of hyposmolality on I_{sc}, but not only due to the inhibitory action on the basal I_{sc} (not due to proportional inhibition to the basal I_{sc}). Since BFA blocks translocation of proteins from endoplasmic reticulum (ER) to the Golgi apparatus [37], it is suggested that the stimulatory action of hyposmolality on I_{Na} is mediated through an increase in number of Na^+ channels in the apical membrane, although we have to consider other possibilities due to side or indirect effects of BFA on the I_{Na} (*see* Discussion in detail). Further, we tested the effect of BFA on the hyposmolality-induced I_{sc} after the I_{sc} had reached its maximum value (approximately 2 hr after cells were exposed to a hyposmotic solution). In this case, BFA had no significant effect on I_{sc}, suggesting that the step of the channel translocation (at least a BFA-sensitive process) stimulated by hyposmolality would be completed when the I_{sc} reaches its maximum value.

Hyposmolality-Induced Increase in Membrane Capacitance via a PTK-Dependent Pathway

So far we showed that hyposmotic stress stimulated Na^+ transport by mainly increasing the number of conducting amiloride-blockable Na^+ channels in the apical membrane. The increase in number of conducting Na^+ channels in the apical membrane may be caused by hyposmolality-mediated translocation of Na^+ channel proteins from intracellular store sites into the apical membrane. To examine a role of PTK in the membrane fusion, which may be affected by hyposmolality in A6 cells like a mammalian urinary bladder [23], accounting for insertion of membrane proteins such as Na^+ channel proteins

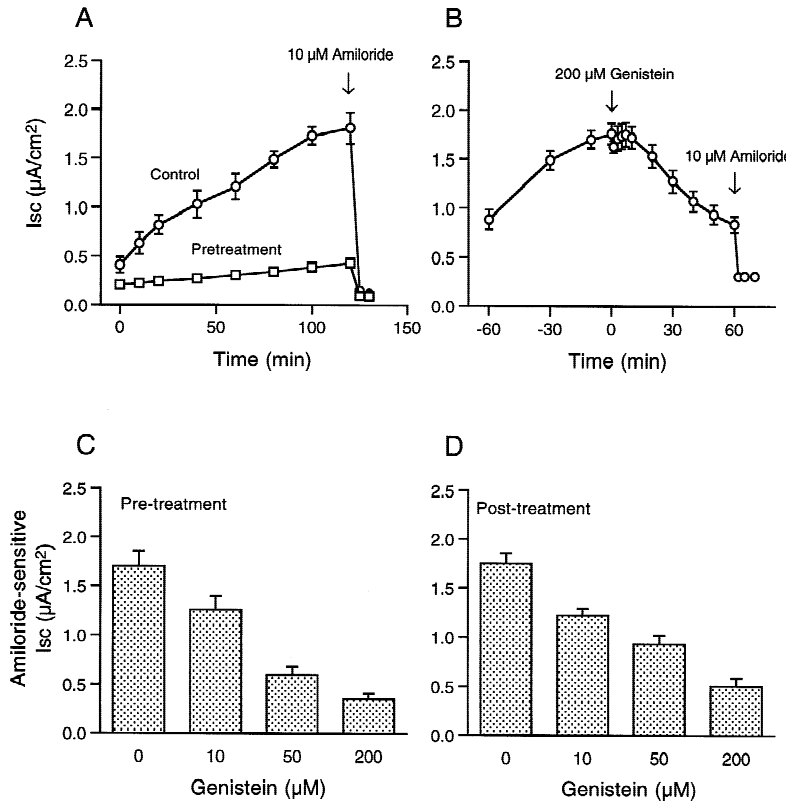

Fig. 7. Effects of genistein on hyposmolality-induced I_{Na}. (*A*) Effect of pretreatment with genistein on hyposmolality-induced I_{Na}. The I_{Na} was measured in A6 cells in a hyposmotic solution of 135 mOsm/kg H_2O without (circles) and with (squares) 200 μM genistein (bilateral pretreatment for 60 min and presence during the experimental period shown in the figure). $n = 6$–8. (*B*) Effect of post-treatment with genistein on the hyposmolality-induced I_{Na}. After the I_{sc} reached a sustained phase in a hyposmotic solution of 135 mOsm/kg H_2O, 200 μM genistein was added to the bilateral sites. $n = 5$. (*C*) Dose-dependent effects of genistein pretreatment on the hyposmolality-induced I_{Na} ($n = 6$–8). The protocol of the experiment was the same as that shown in Fig. 7*A*. (*D*) Dose-dependent effects of genistein post-treatment on the hyposmolality-induced I_{Na} ($n = 5$–8). The protocol of the experiment was the same as that shown in Fig. 7*B*.

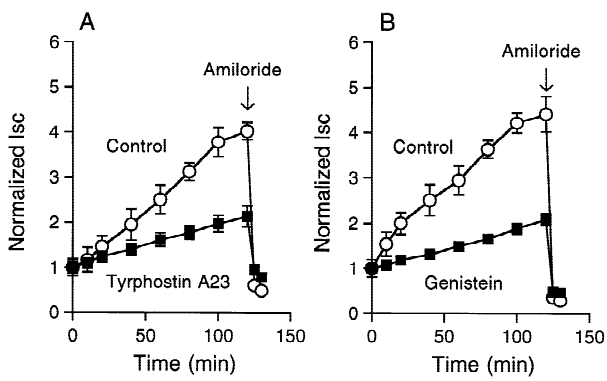

Fig. 8. Effects of PTK inhibitors on normalized I_{sc} induced by hyposmolality. (*A*) The normalized I_{sc} in the presence (closed squares) and absence (control, open circles) of 100 μM tyrphostin A23. (*B*) The normalized I_{sc} in the presence (closed squares) and absence (control, open circles) of 200 μM genistein. Tyrphostin A23 and genistein had been applied to cells 60 min before cell were exposed to a hyposmotic solution. Further, tyrphostin A23 and genistein were also present after cells were exposed to a hyposmotic solution. $n = 5$–8.

into the apical membrane, we measured membrane capacitance (C_T). Hyposmolality (135 mOsm/kg H_2O) caused a rapid increase in C_T which reached a peak 10 min after exposure of cells to a hyposmotic solution. Then, C_T decreased gradually for over 60 min after incubation in a hyposmotic solution (Fig. 12), however the C_T at 60 min was much larger than its basal level under an isosmotic condition. The time course of C_T shown here was very different from that of the I_{Na} (*see* argument about the different time courses in Discussion in detail). On the other hand, a PTK inhibitor, tyrphostin A23 (100 μM), which was applied to the cell for 60 min prior to exposure to a hyposmotic solution and subsequently was present after exposure to a hyposmotic solution, markedly inhibited the hyposmolality-induced increase in C_T (Fig. 12). Interestingly, during the period of exposure to a hyposmotic solution, in tyrphostin A23-treated cells we could not observe a decrease in C_T which was observed in control and BFA-treated cells (*see below*) after C_T reached a peak value (Fig. 12). BFA also diminished an increase in C_T by hyposmolality in a different manner. Namely, BFA (5 μg/ml; pretreatment for 60 min prior to exposure to a hyposmotic solution and subsequent treatment in a hyposmotic solution) also diminished the increase in C_T elicited by hyposmolality (Fig. 12), although the inhibitory effect of BFA on the initial, rapid increase in C_T was smaller than that of tyrphostin A23 (Fig. 12). A decrease in C_T following an initial, rapid increase was observed in BFA-treated cells similar to control, however BFA-treated cells showed a faster decrease in C_T than control (Fig. 12). These results suggest that the activation of PTK by hyposmolality may contribute to the process in the increase in C_T and that the sustained increase in C_T may require to continuous supply of membrane vesicles via a BFA-sensitive pathway.

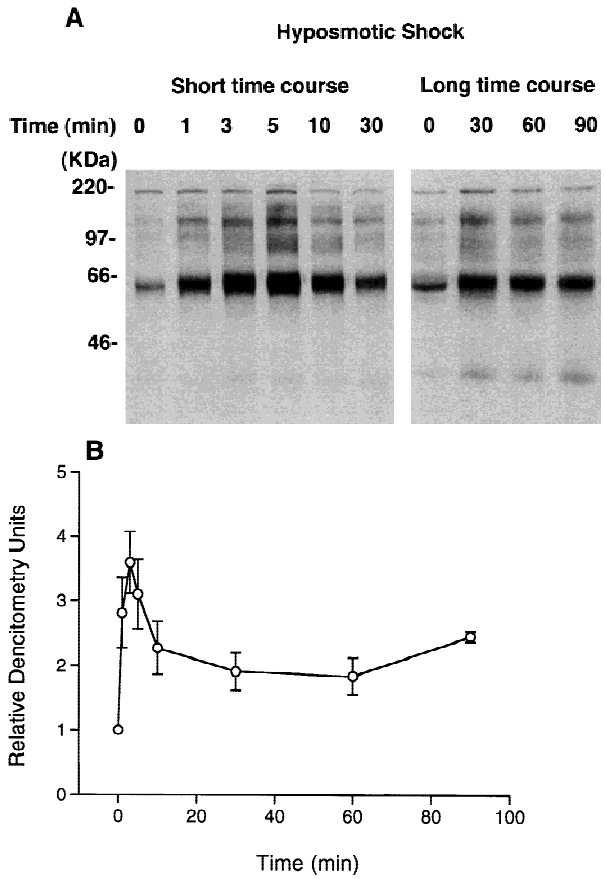

Fig. 9. Effects of hyposmolality on protein tyrosine phosphorylation. Monolayered A6 cells cultured on permeable membrane were subjected to a hyposmotic solution of 135 mOsm/kg H_2O for 0, 1, 3, 5, 10, 30, 60 and 90 min, and 25 μg cell lysate in each lane was then separated by 10% SDS-PAGE and analyzed by immunoblotting with an anti-phosphotyrosine monoclonal antibody (PY99). (*A*) Immunoblotting data of tyrosine phosphorylation by hyposmotic stress for short and long time courses. (*B*) Quantified results of proteins around 60 kDa with imaging densitometer. $n = 6$–9 individual experiments.

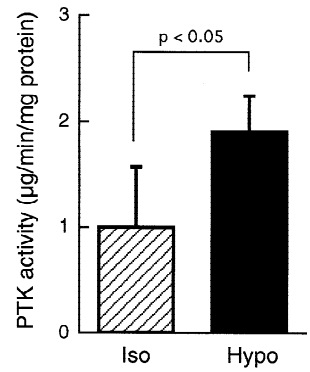

Fig. 10. Effects of hyposmolality on activity of protein tyrosine kinase (PTK). The activity of PTK was measured at 30 min after incubation of cells in an isosmotic or a hyposmotic solution. Hyposmolality increased the activity of PTK about 2–fold ($n = 3$, $P < 0.05$).

Discussion

Osmolality and Na^+ Transport

It is physiologically important to study the regulation of ion transport, especially Na^+ transport, in renal epithelial A6 cells by a change in extracellular osmolality, since the renal epithelium is routinely exposed to various extracellular osmolalities. An increase in the plasma osmolality stimulates osmoreceptors that generate afferent signals calling for the secretion of ADH, leading to antidiuresis by increasing water permeability of the apical membrane of the distal nephron. Conversely, a decrease in the plasma osmolality leads to a decreased concentration of ADH in the blood, resulting in water diuresis by decreased apical water permeability of the distal nephron. In addition, under a hyposmotic condition an increase in active Na^+ reabsorption [27, 43] should occur as well as a decrease in water permeability to immediately recover the plasma osmolality. We indicate in the present study that hyposmolality increased amiloride-sensitive Na^+ transport by increasing the number and activity (open probability) of conducting Na^+ channels at the apical membrane via activation of PTK in a renal epithelial cell line, A6 cell. These phenomena have physiological meanings to maintain the homeostasis of the plasma osmolality.

Osmolality and PTK

In distal nephron epithelial A6 cells, hyposmotic stress induced rapid activation of PTK. Exposure of cells to a hyposmotic solution, in general, causes rapid cell swelling and subsequently reduces their cell volume by loss of various types of solutes, especially K^+ and Cl^- [2, 7, 16, 62]. Cell swelling also causes a change in membrane tension. The changed membrane tension may affect functional activity of membrane-bound proteins such as enzymes by changing their conformation or association each other. A recent study [52] has reported that chlorpromazine, which is known to cause membrane deformation, can mimic the hyposmotic stress-increased tyrosine phosphorylation and c-fos expression in cardiac myocytes. This report [52] also suggests that the cell swelling-induced increase in membrane tension may be the first signal to respond to a change in extracellular osmolality and play a key role in the PTK activation. However, it is still unclear whether PTK (membrane bound) directly senses the membrane tension. Rosette and Karin [50] have reported that osmotic (hyperosmotic) stress and ultraviolet irradiation strongly activate the c-Jun amino-terminal protein kinase (JNK) cascade which is caused by clustering and internalization of cell

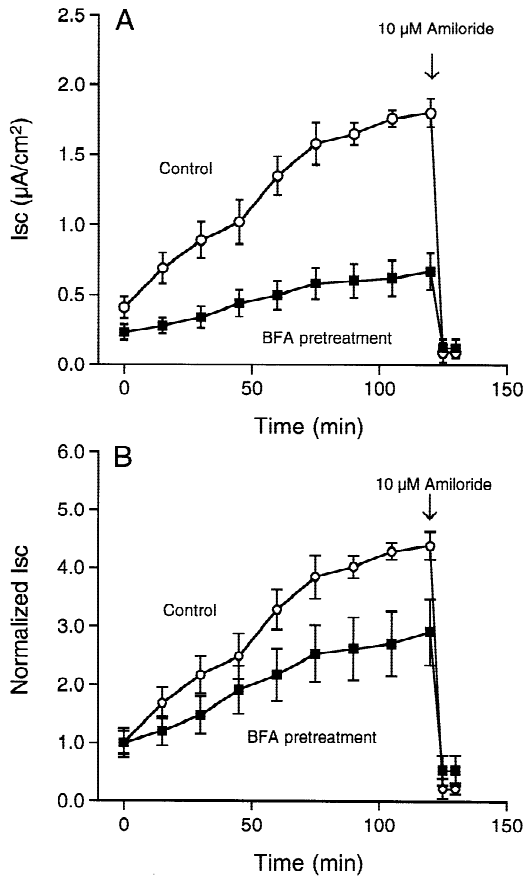

Fig. 11. Effects of BFA on hyposmolality-induced I_{sc}. (*A*) **Control** (open circles): Cells were exposed to a hyposmotic solution of 135 mOsm/kg H_2O at 0 min from an isosmotic solution of 255 mOsm/kg H_2O. $n = 5$. **BFA** (closed squares): Cells were pretreated with brefeldin A (BFA; 5 μg/ml; bilateral application) in an isosmotic solution of 255 mOsm/kg H_2O for 60 min and subsequently exposed to a hyposmotic solution of 135 mOsm/kg H_2O containing 5 μg/ml BFA from 0 min during the period of the I_{sc} measurement. (*B*) Normalized I_{sc} in the presence and absence of BFA. The normalized I_{sc} in the presence (closed squares) and absence (control, open circles) of BFA (5 μg/ml; bilateral application). $n = 3$.

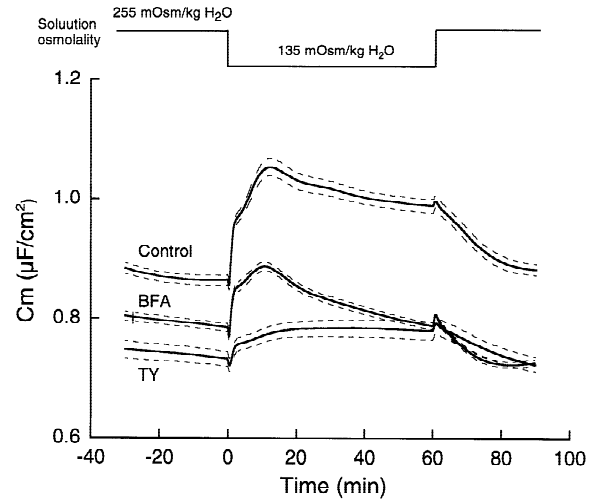

Fig. 12. Hyposmolality-induced increase in membrane capacitance via a PTK-dependent pathway. For maintenance of epithelial polarities, A6 cells were cultured on permeable membrane. The membrane capacitance was measured in polarized A6 cells. **Control:** Cells were incubated in an isosmotic solution of 255 mOsm/kg H_2O and subsequently exposed to a hyposmotic solution of 135 mOsm/kg H_2O at 0 min. **TY:** Cells were pretreated with tyrphostin A23 (TY; 100 μM) in an isosmotic solution of 255 mOsm/kg H_2O for 60 min and subsequently exposed to a hyposmotic solution of 135 mOsm/kg H_2O containing 100 μM tyrphostin A23 at 0 min. **BFA:** Cells were pretreated with brefeldin A (BFA; 5 μg/ml) in an isosmotic solution of 255 mOsm/kg H_2O for 60 min and subsequently exposed to a hyposmotic solution of 135 mOsm/kg H_2O containing 5 μg/ml BFA at 0 min. $n = 4$ for each experiment. Solid and dash lines, respectively, express the mean and SE values under each experimental condition.

surface receptors for epidermal growth factor (EGF), tumor necrosis factor (TNF), and interleukin-1 in HeLa cells. This report [50] suggests that physical stresses by hyperosmotic shock may perturb the cell surface or alter receptor conformation and that the initial signaling event activating the JNK cascade in response to hyperosmotic stress is multimerization and clustering of cell surface receptors for growth factors and cytokines. The structure of the plasma membrane itself may regulate functions of membrane-bound proteins such as cell surface receptors, ion transporters and protein kinases by changing conformation of proteins through changes in membrane tension and/or other physical stress [45]. In A6 cells, hyposmotic shock causes initial, transient cell swelling followed by RVD, subsequently almost recovering to initial cell volume [10, 11].

Time Courses of Na^+ Transport and Tyrosine Phosphorylation Induced by Hyposmotic Stress

There is a question on the time courses of a change in cell volume and stimulation of Na^+ transport. The change in cell volume was transient, while stimulation of Na^+ transport continued over 120 min. There are at least two possibilities explaining the different time courses of the hyposmolality-induced changes in cell volume and Na^+ transport: (i) one is that the transient increase in membrane tension by swelling is a large enough signal to turn the initial switch on for activation of downstream signaling leading to stimulation of Na^+ transport, and (ii) the other is that cytosolic ion environments, especially Cl^- concentration, may affect the regulatory mechanism to stimulate Na^+ transport, since it is expected that the process of RVD reduces the cytosolic Cl^- concentration [27, 30] which affects the activity of protein kinases [20] and ion channels [18, 56].

Observations from some studies on the signaling pathway of hyposmolality [52, 55] might explain our experimental results as follows: (i) activation of PTK occurs in the initial phase of the hyposmotic stress, (ii) the hyposmolality-activated PTK increases the number and activity of conducting Na^+ channels at the apical membrane, and (iii) since the effects of PTK have been transduced to the effector (i.e., Na^+ channels), PTK inhibitors should not have any effects if the inhibitor is applied after hyposmotic stress. In the present study, the hyposmolality-induced I_{Na} was diminished by PTK inhibitors (tyrphostin A23 and genistein) that were applied even after hyposmotic stress had increased the I_{Na}. This observation seems to be inconsistent with the signaling pathway described above based on the observations by Tilly et al. [55] and Sadoshima et al. [52]. However, as shown in Fig. 9, hyposmotic stress increased phosphotyrosine over 90 min after exposure to hyposmolality. This observation suggests that even when the I_{Na} reached a sustained level, PTK activity would be still maintained at a higher level than the basal one. The higher activity of PTK would be required to continuously stimulate the Na^+ transport, and the inhibition of the increased PTK activity by PTK inhibitors would result in the diminution of the Na^+ transport even when the PTK inhibitor was applied after hyposmotic stress had increased the Na^+ transport. Taken together, these observations suggest two roles of PTK: (i) the initial activation of PTK plays a role as a trigger in the hyposmolality-induced downstream signaling, and (ii) the sustained activation of PTK maintains continuous stimulation of Na^+ transport.

Tyrosine-Phosphorylation Induced by Hyposmotic Stress

In this study, we detected increases in tyrosine-phosphorylation in multiple proteins after exposure of A6 cells to a hyposmotic solution. By immunoblotting with a monoclonal anti-phosphotyrosine antibody (PY99), a major band was detected as ~60 kDa protein(s). The contents of phosphotyrosine in this band increased about 4-fold within 5 min and were maintained at an increased level higher than the basal one over 90 min after exposure of cells to hyposmolality. As shown in our previous report [44], tyrphostin A23 diminished tyrosine phosphorylation of proteins caused by hyposmolality, indicating that a PTK inhibitor, tyrphostin A23, inhibited PTK activity. These results indicate that PTK activation rapidly would occur after exposure to a solution with hyposmolality. Other minor bands, ~40, ~80, ~110–120 kDa proteins, also increased in the content of tyrosine phosphorylation. These proteins might also contribute to the increase in number and activity of conducting Na^+ channels induced by hyposmolality, although we need further studies to determine what types of PTK are involved in the regulation of Na^+ transport by hyposmolality to clarify the signaling process of osmolality to stimulation of Na^+ transport in more detail.

Other Possible Regulatory Pathways of Amiloride-Sensitive Na^+ Transport by Hyposmotic Stress

In the present study, we indicate that activation of PTK is involved in stimulatory pathways of amiloride-sensitive Na^+ transport by hyposmotic stress. In addition to this pathway, other pathways would also be involved in the hyposmolality-induced stimulation of amiloride-sensitive Na^+ transport. A recent study [36] suggests that in A6 cells, prostaglandin E_2 mimics the stimulatory action of hyposmotic stress on the Na^+ transport and inhibitors of prostaglandin E_2 synthesis such as indomethacin, quinacrine and 3[4-octadecyl]-benzoylacrylic acid block the stimulatory action of hyposmotic stress on the Na^+ transport. The study [36] clearly indicates that prostaglandin E_2 plays a role in stimulatory regulation of the Na^+ transport, and that hyposmotic stress has no additive effects on the action of prostaglandin E_2, concluding that prostaglandin E_2 is a mediator of the hyposmotic action on the Na^+ transport in A6 cells. However, unfortunately the study [36] dose not report whether hyposmotic stress stimulates synthesis of prostaglandin E_2 in A6 cells. This implies that although the stimulatory action of prostaglandin E_2 on the Na^+ transport in A6 cells is confirmed, it is still unknown whether hyposmotic stress stimulates the Na^+ transport by increasing synthesis of prostaglandin E_2. In other words, it might be possible that prostaglandin E_2 plays a role in stimulation of the Na^+ transport as a parallel pathway to the signaling pathway of hyposmolality, and that prostaglandin E_2 only shares an effector (i.e., amiloride-sensitive Na^+ channels) with hyposmotic stress, resulting in no additive action on the amiloride-sensitive Na^+ transport. To clarify the role of prostaglandin E_2 in the hyposmolality-induced signaling pathway to stimulation of amiloride-sensitive Na^+ transport, we need further experimental observations.

Effects of PTK Inhibitors on the Na^+, K^+-Pump

Amiloride-sensitive transepithelial Na^+ transport is mediated through two steps; (i) a Na^+ entry step through amiloride-sensitive Na^+ channels at the apical membrane, and (ii) a Na^+ extrusion step by the Na^+,K^+-pump at the basolateral membrane [27]. It is, in general, thought that the entry step of Na^+ across the apical membrane is the rate-limiting step in transepithelial Na^+ trans-

port (*see reviews* [14, 27]). Indeed, in A6 cells, the entry step of Na^+ across the apical membrane is the rate limiting one in transepithelial Na^+ transport under the basal condition [26, 27], suggesting that an increase in Na^+ entry is required to stimulate the transepithelial Na^+ transport. On the other hand, inhibition of the Na^+ transport is under a situation different from stimulation of the Na^+ transport; i.e., if either of these two steps is blocked, the amiloride-sensitive transepithelial Na^+ transport is abolished.

Our previous report [44] indicates that a PTK inhibitor, tyrphostin A23 (100 μM), diminished the Na^+,K^+-pump activity. Therefore, the inhibitory action of tyrphostin A23 on I_{Na} shown in the present study would be due to inhibition of the Na^+,K^+-pump activity, although we show the inhibitory action of tyrphostin A23 on the channel number and activity of the 4-pS amiloride-sensitive Na^+ channel. However, the current generated by the Na^+,K^+-pump exceeded the I_{Na} even in tyrphostin A23-treated cells [44], indicating that under the condition that tyrphostin A23 diminishes the transepithelial Na^+ transport, the rate-limiting step is still the Na^+ entry step through amiloride-sensitive Na^+ channels. Therefore, the inhibition of I_{Na} by tyrphostin A23 shown in the present study is really caused by the inhibitory action of tyrphostin A23 on the Na^+ channel (the Na^+ entry step).

When the Na^+,K^+-pump is inhibited, the cytosolic Na^+ concentration ($[Na^+]_c$) would increase. This increase in $[Na^+]_c$ would affect the cytosolic ion environments including the cytosolic Ca^+ concentration and pH, which would affect the Na^+ channel activity. Therefore, we should consider a possibility of the secondary effect due to inhibition of the Na^+,K^+-pump by PTK inhibitors [44].

Effects of PTK Inhibitors and BFA on the Hyposmolality-Induced I_{sc}

When BFA was applied to cells after the I_{sc} had reached its maximum value in a hyposmotic solution, BFA had no significant effects on the I_{sc}. On the other hand, PTK inhibitors, tyrphostin A23 and genistein, had still inhibitory action on the hyposmolality-induced I_{sc} even after the I_{sc} reached its maximum value. These observations suggest that the translocation step (at least the BFA-sensitive step) of the 4-pS Na^+ channel to the apical membrane would be completed when the I_{sc} reaches its maximum value in a hyposmotic solution (approximately 2 hr after the cell is exposed to a hyposmotic solution), and that a PTK-dependent pathway would be still involved in the regulation of the 4-pS Na^+ channel even after the I_{sc} reaches its maximum value in a hyposmotic solution.

Action of BFA on Protein Trafficking

ADP ribosylation factors (ARFs), which are GTPases, regulate intracellular vesicular membrane trafficking [39, 53]. Guanine nucleotide-exchange proteins (GEPs) activate ARFs by stimulating the replacement of bound GDP with GTP [39, 48, 53]. BFA, which is known to inhibit GEP activity of ARF in Golgi membranes, causes reversibly apparent dissolution of the Golgi complex in many cells [12], blocking intracellular trafficking of vesicular membrane containing proteins [4, 8]. In the present study, we indicate that hyposmolality stimulated Na^+ transport in A6 cells and that the process of stimulation of Na^+ transport was mainly due to an increase in number of conducting Na^+ channels in the apical membrane. BFA inhibited the stimulatory action of hyposmolality on conducting Na^+ channels. These results suggest that hyposmolality would increase Na^+ transport by stimulation of Na^+ channel trafficking to the apical membrane, although we should also consider another possibility that BFA would abolish the hyposmolality-induced activation of Na^+ transport by blocking translocation of regulatory proteins (not the channel protein itself) to the apical membrane which activate nonconducting Na^+ channels pre-existing at the apical membrane.

The Action of PTK Inhibitor and BFA on Hyposmotic Stress-induced Changes in C_T

Hyposmolality caused a rapid increase in C_T. The interesting point is that a PTK-dependent process is involved in the increase in C_T by hyposmolality, since tyrphostin A23, a PTK inhibitor, significantly inhibited the increase in C_T. On the other hand, BFA affected the change in C_T caused by hyposmolality was still observed in the presence of BFA, although the magnitude of the transient increase was smaller than that observed in control. However, the sustained phase in the presence of BFA decreased more rapidly than that in control. These results suggest that BFA does not inhibit the process in exocytotic insertion of membrane vesicles locating at submembrane areas which might contribute to the initial, rapid increase in C_T, and that the sustained increase in C_T requires new membrane vesicles continuously supplied from intracellular sites which are far from the apical membrane via a BFA-dependent pathway.

Time Courses of Na^+ Transport and C_T Induced by Hyposmotic Stress

The time course of the change in C_T by hyposmotic stress was very different from that in I_{Na}. This would be due to the hyposmolality-induced incorporation of various

types of membrane vesicles containing various other types of proteins in addition to amiloride-sensitive Na^+ channels into the apical membrane, since the stimulation of Na^+ transport is just one of multiple phenomena caused by hyposmotic stress. The insertion of membrane vesicles containing other proteins into the apical membrane with a time course different from that in I_{Na} would also contribute to adaptation for changes in extracellular environment.

Osmosensors

Two types of osmosensors independently found in yeast regulate a common osmosensing HOG MAP kinase cascade in response to the change of extracellular osmolality [25, 49]. SLN1, a putative osmosensor, is homologous to the prokaryotic two-component system which has a sensor molecule containing a cytoplasmic histidine kinase domain. Recognition of environmental changes by sensor molecules causes either activation or inhibition of its histidine kinase domain. Low osmolality of medium activates the SLN1 histidine kinase. These reports [25, 49] suggest that sensor molecules in osmosensor may recognize directly the change in osmolality. Removal of solutes for reduction of osmolality elicits changes in ion strength and specific ion concentration. In some cases, these conditions in bathing solutions may affect enzyme activity and protein conformation. The sensor molecule in yeast SLN1 may recognize ion strength or ion concentration which affects histidine kinase activity, transducing signaling pathways involved in changes of osmolality.

Conclusion

In summary, the present study indicates that (i) hyposmotic stress increases the PTK activity and amount of phosphotyrosine, (ii) hyposmotic stress increases I_{Na} by elevating the number and activity (open probability) of conducting Na^+-channels in the apical membrane via a BFA-sensitive, PTK-dependent pathway, (iii) hyposmotic stress increases C_T in a PTK-dependent pathway. As a conclusion, it is suggested that hyposmolality would stimulate the translocation of Na^+ channel (or regulatory) proteins from intracellular store sites to the apical membrane via activation of PTK in a BFA-sensitive pathway and the process in translocation of Na^+ channel (or regulatory) proteins to the apical membrane and/or fusion of vesicle containing Na^+ channel (or regulatory) proteins mediated via the hyposmolality-induced PTK activation.

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