Hypotonicity stimulates renal epithelial sodium transport by activating JNK via receptor tyrosine kinases

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Taruno A, Niisato N, Marunaka Y. Hypotonicity stimulates renal epithelial sodium transport by activating JNK via receptor tyrosine kinases. Am J Physiol Renal Physiol 293: F128–F138, 2007. First published March 6, 2007; doi:10.1152/ajprenal.00011.2007.—We previously reported that hypotonic stress increased transepithelial Na+ (Na+)-K+-ATPase activity via a pathway dependent on protein tyrosine kinase (PTK; Niisato N, Van Driessche W, Liu M, Marunaka Y. J Membr Biol 175: 63–77, 2000). However, it is still unknown what type of PTK mediates this stimulation. In the present study, we investigated the role of receptor tyrosine kinase (RTK) in the hypotonic stimulation of Na+ transport. In renal epithelial A6 cells, we observed inhibitory effects of AG1478 [an inhibitor of the EGF receptor (EGFR)] and AG1296 [an inhibitor of the PDGF receptor (PDGFR)] on both the hypotonic stress-induced stimulation of Na+ transport and the hypotonic stress-induced ligand-independent activation of EGFR. We further studied whether hypotonic stress activates members of the MAP kinase family, ERK1/2, p38 MAPK, and JNK/SAPK, via an RTK-dependent pathway. The present study indicates that hypotonic stress induced phosphorylation of ERK1/2 and JNK/SAPK, but not p38 MAPK, that the hypotonic stress-induced phosphorylation of ERK1/2 and JNK/SAPK was diminished by coinhibition of AG1478 and AG1296, and that only JNK/SAPK was involved in the hypotonic stimulation of Na+ transport. A further study using cyclohexamide (a protein synthesis inhibitor) suggests that both RTK and JNK/SAPK contributed to the protein synthesis-independent early phase in hypotonic stress-induced Na+ transport, but not to the protein synthesis-dependent late phase. The present study also suggests involvement of phosphatidylinositol 3-kinase (PI3-kinase) in RTK-JNK/SAPK cascade-mediated Na+ transport. These observations indicate that 1) hypotonic stress activates JNK/SAPK via RTKs in a ligand-independent pathway, 2) the RTK-JNK/SAPK cascade acts as a mediator of hypotonic stress for stimulation of Na+ transport, and 3) PI3-kinase is involved in the RTK-JNK/SAPK cascade for the hypotonic stress-induced stimulation of Na+ transport. ENaC; epidermal growth factor

REGULATION OF Na+ TRANSPORT in the distal nephron of the kidney plays a key role in maintenance of extracellular fluid volume. A wide variety of regulatory factors such as aldosterone, insulin, vasopressin (antiuretic hormone), and osmotic stress have been reported to be involved in the regulation of the renal functions. Transepithelial Na+ transport is carried out in a two-step process: 1) Na+ entry into the cytosolic space across the apical membrane through the amiloride-sensitive epithelial Na+ channel (ENaC) and 2) Na+ extrusion from the cytosolic space across the basolateral membrane through the Na+–K+–ATPase. Under some conditions that the Na+ extrusion by the Na+–K+–ATPase becomes the rate-limiting step, many factors are recognized to control Na+ transport by regulating the Na+–K+–ATPase, and activation of the regulatory factors is a prerequisite to increase Na+ transport (28, 37). However, since the Na+ entry step is, in general, recognized as the rate-limiting step for transepithelial Na+ transport, ENaC is the target of many regulatory factors for Na+ transport.

Although many studies performed in several laboratories (12, 21, 22, 28, 32, 39, 54) have reported the stimulatory mechanism of Na+ transport in renal epithelial A6 cells in response to changes in osmolality, it still remains poorly understood. Exposure of the basolateral, but not apical, membrane of A6 cells to hypotonicity increases the transepithelial Na+ transport mainly by increasing the number of conductive ENaCs at the apical surface (39). Our previous work demonstrated that hypotonicity-induced activation of protein tyrosine kinases (PTK) played a crucial role in the hypotonicity-induced activation of Na+ transport (39). However, we have no information on what type of PTK acts on the hypotonicity-induced activation of Na+ transport. In the present study, we aimed to identify what type of PTK contributes to the hypotonic action.

UV light or osmotic stress strongly activates the JNK/SAPK through multiple growth factor and cytokine receptors (42). Exposure to UV light or osmotic stress induces clustering and internalization of cell surface receptors for EGF, tumor necrosis factor α, interleukin-1, and possibly other growth factors and cytokines, independently of their specific ligand binding. In general, EGF leads to JNK/SAPK activation via a pathway dependent on activation of the small GTP-binding proteins Ras (34) and Rac (34) and the MAPK kinase kinase MEKK1 (35). In turn, MEKK1 phosphorylates and activates the MAPK kinase MKK4, which activates JNK/SAPK (13, 44).

Notably, growth factors such as EGF and IGF have been recognized as stimulants of Na+ transport in A6 cell monolayers (2, 3, 27), and growth factor receptors themselves could act as a receptor-type protein tyrosine kinase (RTK). Therefore, we hypothesized that hypotonicity shows its stimulatory action on Na+ transport by activating RTK without any ligand binding. Furthermore, our earlier work (38) and others (9) have indicated that hypotonic stress activates members of the MAPK family, such as JNK/SAPK, p38 MAPK, and ERK1/2, in A6 cells. Based upon these observations, we predicted more that the hypotonicity induces activation of JNK/SAPK via RTK leading to stimulation of Na+ transport.

In the present study, we report that RTK inhibitors dose dependently diminished the hypotonicity-induced Na+ transport and the ligand-independent activation of EGFR by hypo-
tonic stress. Furthermore, we found that hypotonicity-induced activation of JNK/SAPK was mediated through RTKs and was involved in the activation of Na\(^+\) transport by hypotonic stress. These results indicate that ligand-independent activation of RTKs by hypotonic stress leads to JNK/SAPK activation, resulting in the elevation of Na\(^+\) transport. Finally, we studied the possible activation of the phosphatidylinositol 3-kinase (PI3-kinase) pathway by hypotonicity and report here that PKB, the phosphorylation (activation) of which is mediated by PI3-kinase, was phosphorylated at both Thr 308 and Ser 473 by hypotonic stress through the RTK-JNK/SAPK cascade, indicating that hypotonicity activates PI3-kinase via the RTK-JNK/SAPK cascade, leading to stimulation of Na\(^+\) transport.

**MATERIALS AND METHODS**

**Chemicals and materials.** AG1478, AG1296, LY294002, U0126, SB203580, and JNK Inhibitor II were obtained from Calbiochem (San Diego, CA). Fetal bovine serum was purchased from Cambrex Bio Science (Walkersville, MD). Permeable tissue culture supports (Nunc Tissue Culture Inserts) were obtained from Nunc (Roskilde, Denmark). NCTC-109 medium, benzamil, and cycloheximide were purchased from Sigma (St Louis, MO).

**Solutions.** The isotonic solution (255 mosmol/kg H\(_2\)O) contained (in mM) 95 NaCl, 3.5 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 25 NaHCO\(_3\), 10 HEPES, and 5 glucose. The hypotonic solution (135 mosmol/kg H\(_2\)O) contained (in mM) 35 NaCl, 3.5 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 25 NaHCO\(_3\), 10 HEPES, and 5 glucose. The pH of solutions used in the present study was adjusted to 7.4 by NaOH before addition of NaHCO\(_3\).

**Cell culture.** Renal epithelial A6 cells derived from *Xenopus laevis* were purchased from American Type Culture Collection (Rockville, MD) at passage 68. A6 cells (passages 73–84) were grown on plastic flasks at 27°C in a humidified incubator with 1.0% CO\(_2\) in air in a culture medium which contained 75% (vol/vol) NCTC-109, 15% (vol/vol) distilled water, and 10% (vol/vol) fetal bovine serum. Cells were seeded onto Nunc tissue culture inserts (Nunc, Roskilde, Denmark) for Western blotting or onto tissue culture-treated Transwell filter cups (Costar, Cambridge, MA) for electrophysiological measurements at a density of 5 × 10\(^4\) cells/well and were cultured for 11–15 days.

**Measurement of short-circuit current.** Monolayers of A6 cells subcultured on tissue culture-treated Transwell filter cups were transferred to a modified Ussing chamber (Jim’s Instrument, Iowa City, IA) designed to hold the filter cup. 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). Permeable tissue culture supports (Nunc Tissue Culture Inserts) were obtained from Nunc (Roskilde, Denmark). NCTC-109 medium, benzamil, and cycloheximide were purchased from Sigma (St Louis, MO).

**Data presentation.** All data are presented as means ± SE. Where SE bars are not visible, they are smaller than the symbol. Student’s *t*-test and ANOVA were used for statistical analysis as appropriate, and *P* < 0.05 was considered significant.

**RESULTS**

Hypotonic stress-induced increases in Na\(^+\) transport and Na\(^+\) conductance in an RTK-dependent manner. In the present study, we applied hypotonic stress bilaterally to A6 cells by reducing the osmolality from 255 to 135 mosmol/kg H\(_2\)O with removal of 60 mM NaCl. Exposure of A6 cells to a hypotonic solution increased the *I*\(_{sc}\) as shown in Fig. 1. *G*\(_i\) was also increased by hypotonic stress similar to *I*\(_{sc}\), and the time course of the increase in *G*\(_i\) is the same as that in *I*\(_{sc}\) (data not shown). Most of the increase in *I*\(_{sc}\) was sensitive to 10 μM benzamil, which is a derivative of amiloride and had ~10 nM of the concentration showing the half-maximum inhibition (IC\(_{50}\)) to the hypotonicity-induced *I*\(_{sc}\) and *G*\(_i\) (data not shown). The IC\(_{50}\)
measurements of AG1478 was present in the solution during the period for A6 cells 60 min before exposure to hypotonicity, and Na
Therefore, we studied roles of RTKs in hypotonicity-increased protein tyrosine kinase localized in the basolateral membrane, and interestingly growth factor receptors themselves act as a functional ENaC at the apical membrane via a PTK-dependent manner. However, it remains unknown what type of PTK is obtained in the present study is identical to the IC50 obtained at a single-channel current level of an amiloride-sensitive 4-pS highly Na+-selective channel (i.e., ENaC) (31), which is the predominant amiloride-sensitive Na+-permeable channel contributing to transepithelial Na+ transport in A6 monolayer (29, 30). These results suggest that the hypotonic stress increases I\textsubscript{sc} and G\textsubscript{f} and that the increased I\textsubscript{sc} is Na+ transport through benzamil-sensitive ENaCs in the apical membrane.

Our previous work (39) reported that hypotonicity stimulated Na+ transport mainly by increasing the number of functional ENaC at the apical membrane via a PTK-dependent manner. However, it remains unknown what type of PTK is involved in the stimulatory action of hypotonicity on Na+ transport. Recently, growth factors such as IGF (2, 3) and EGF (27) have been recognized as the stimulants of Na+ transport. We examined an effect of another RTK inhibitor (AG1296, an inhibitor of PDGFR). AG1296 also showed inhibitory effects on the hypotonic stress-induced I\textsubscript{Na} and G\textsubscript{Na} in a dose-dependent manner (Fig. 1, B and D). The inhibitory action of AG1296 would reach its maximum level at ~10 \mu M. The study on dose-dependent action of AG1296 indicates that the IC50 was ~3 \mu M. Furthermore, coapplication of AG1478 (10 \mu M) and AG1296 (10 \mu M) showed a significantly larger inhibitory effect on the hypotonic stress-induced I\textsubscript{Na} and G\textsubscript{Na} compared with the application of AG1478 or AG1296 alone (Fig. 2), suggesting that both EGFR and PDGFR are involved in the stimulatory action of hypotonic stress on Na+ transport, although we should consider a possible involvement of RTKs other than EGFR and PDGFR.

Transactivation of EGFR by hypotonic stress. Although the observations shown in Fig. 1 indicate the hypotonic stress shows a part of its stimulatory action on I\textsubscript{Na} and G\textsubscript{Na} via EGFR, we have no information on whether the hypotonic stress activates EGFR. To clarify this point, we studied whether the hypotonic stress elevates the Tyr 845-phosphorylated level of EGFR, the activated form of EGFR (1, 11, 20). Under the isotonic condition, a low level of phosphorylation of EGFR on Tyr 845 was observed (Fig. 3A). Hypotonic stress stimulated phosphorylation of EGFR in a time-dependent manner (Fig. 3A); the hypotonicity-induced phosphorylation reached a peak and then gradually decreased to the basal level with time. We also studied whether AG1478 affects the hypotonicity-induced phosphorylation of EGFR. A6 cell monolayers were preincu-

**Fig. 1.** Effects of hypotonicity on short-circuit current (I\textsubscript{sc}) in A6 monolayers and suppression of the hypotonicity-induced, benzamil-sensitive I\textsubscript{sc} by receptor tyrosine kinase (RTK) inhibitors. A and B: A6 cell monolayers were preincubated in an isotonic solution containing vehicle (open symbols) or an EGF receptor (EGFR) kinase inhibitor, AG1478 (A), or a PDGFR (PDGF receptor) kinase inhibitor, AG1296 (B), of the indicated concentrations (filled symbols) for 60 min before exposure to the hypotonic solution. Benzamil (10 \mu M) was applied to the apical side at 120 min after exposure to the hypotonic solution. Dose-dependent effects of AG1478 or AG1296 are shown in the time courses of changes in I\textsubscript{sc} (n = 3–8). C and D: effects of AG1478 (C) and AG1296 (D) on the benzamil-sensitive I\textsubscript{sc} and transepithelial conductance (G\textsubscript{f}). AG1478 and AG1296 diminished the benzamil-sensitive I\textsubscript{sc} and G\textsubscript{f} in a dose-dependent manner (n = 3–8).
bated with vehicle or AG1478 (10 \mu M) for 60 min and then exposed to hypotonic stress. As shown in Fig. 3B, AG1478 of 10 \mu M abolished the basal and hypotonicity-induced phosphorylation of EGFR on Tyr 845.

**Activation of ERK1/2 and JNK/SAPK by hypotonic stress via RTK.** Osmotic stress is one of factors activating the MAP kinase family, such as ERK1/2, p38 MAPK, and JNK/SAPK, in several types of cells (43, 50, 53). It has been demonstrated that hypotonic stress activates ERK1/2, p38 MAPK, and JNK/SAPK in A6 cells (9, 38). To investigate the relationship between RTK and the activation of the MAP kinase family by hypotonic stress, we studied the effects of AG1478 and AG1296 on hypotonicity-induced activation of ERK1/2, p38 MAPK, and JNK/SAPK. A6 cell monolayers were preincubated for 60 min in the absence or presence of RTK inhibitors [coapplication of AG1478 (10 \mu M) and AG1296 (10 \mu M)] and then exposed to hypotonic stress (135 mosmol/kg H2O) for up to 120 min. Hypotonic stress increased the levels of phosphorylated forms of ERK1/2, p38 MAPK, and JNK/SAPK; the maximum levels of phosphorylation were observed 5–15 min after exposure to hypotonic stress, and then the levels of phosphorylation were gradually decreased to the basal levels (Fig. 4). The coapplication of AG1478 and AG1296 diminished the basal [ERK1/2 = 0.06 \pm 0.01 of control (DMSO, n = 3, P < 0.001) and JNK/SAPK = 0.22 \pm 0.12 of control (DMSO, n = 3, P < 0.025)] and hypotonic stress-induced phosphorylation of ERK1/2 [0.44 \pm 0.07 of control (DMSO, n = 3, P < 0.025) at 5 min] and JNK/SAPK [0.39 \pm 0.14 of control (DMSO, n = 3, P < 0.05) at 5 min], whereas the inhibition of RTK had no apparent effect on the basal [0.97 \pm 0.48 of control (DMSO, n = 3, not significant)] or hypotonicity-increased levels [1.67 \pm 0.20 of control (DMSO, n = 3, not significant) at 5 min] of phosphorylated p38 MAPK. These results indicate that ERK1/2 and JNK/SAPK are located in the downstream of hypotonic stress-activated RTK signaling pathway.

**Involvement of hypotonic stress-activated RTK-JNK/SAPK signaling pathway in hypotonicity-increased \( I_{Na} \) and \( G_{Na} \).** As described above, hypotonic stress activated ERK1/2, p38 MAPK, and JNK/SAPK. Therefore, we studied whether these signaling pathways participate in the hypotonicity-increased \( I_{Na} \) and \( G_{Na} \). A6 cell monolayers were preincubated for 60 min with vehicle (DMSO), 2 \mu M U-0126 (an inhibitor of ERK1/2), 20 \mu M SB203580 (an inhibitor of p38 MAPK), or 10 \mu M JNK inhibitor II (an inhibitor of JNK/SAPK) and then exposed to hypotonic stress. U-0126 or SB203580 pretreatment had no effects on the \( I_{Na} \) or \( G_{Na} \) increased by hypotonic stress, whereas the preincubation with JNK inhibitor II drastically diminished the hypotonic action on \( I_{Na} \) and \( G_{Na} \) (Fig. 5). As shown in Fig. 6, the inhibitory effect of JNK inhibitor II was significantly larger than that with coapplication of AG1478 and AG1296, and we could observe no additive effect of coapplication of AG1478 and AG1296 on the hypotonicity-increased \( I_{Na} \) in the presence of JNK inhibitor II, suggesting that other types of RTKs in addition to EGFR and PDGFR are located in the upstream of hypotonicity-activated JNK/SAPK and/or that AG1478 or AG1296 incompletely inhibits the RTK. Although we should still consider involvements of other types of RTKs in addition to EGFR and PDGFR in the upstream of hypotonicity-activated JNK/SAPK and/or incomplete inhibition of

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**Fig. 2. Effects of coapplication of AG1478 and AG1296 on hypotonic stress-increased \( Na^+ \) transport.** A: A6 cell monolayers were preincubated in the isotonic solution containing vehicle (DMSO, ○), 10 \mu M AG1478 (●), 10 \mu M AG1296 (△), or coapplication of 10 \mu M AG1478 and 10 \mu M AG1296 (◇) for 60 min before exposure to the hypotonic solution. Benzamil (10 \mu M) was applied to the apical side at 120 min after exposure to hypotonic solution (n = 4). B and C: effects of 10 \mu M AG1478, 10 \mu M AG1296, and coapplication of 10 \mu M AG1478 and 10 \mu M AG1296 on benzamil-sensitive \( I_{Na} \) (B) and \( G_{Na} \) (C) at 120 min after exposure to the hypotonic solution. Coapplication of AG1478 and AG129 showed a significantly larger inhibitory action on hypotonicity-stimulated benzamil-sensitive \( I_{Na} \) and \( G_{Na} \) than that caused by AG1478 or AG1296 alone (n = 4). *P < 0.05.
Figure 8 shows that hypotonic stress transiently stimulated the phosphorylation of EGFR on Tyr 845 with its maximal level observed at ∼5 min. This blot was initially blotted with anti-EGFR antibody (bottom) followed by stripping and reprobing with anti-phospho-EGFR (Tyr 845) antibody (top). B: 10 μM AG1478 abolished the hypotonic stress-induced phosphorylation of EGFR on Tyr 845. A6 cell monolayers were preincubated with vehicle or AG1478 for 60 min and then exposed to the hypotonic solution for the 10 min. This blot was initially blotted with anti-EGFR antibody (bottom) and then stripped and reprobed with anti-phospho-EGFR (Tyr 845) antibody (top). ISO, isotonic condition; HYPO, hypotonic condition.

Figure 3. Ligand-independent phosphorylation of EGFR on Tyr 845 by hypotonic stress and effect of AG1478 on the hypotonic stress-induced phosphorylation of EGFR on Tyr 845. A: hypotonic stress transiently stimulated the phosphorylation of EGFR on Tyr 845 with its maximal level observed at ∼5 min. This blot was initially blotted with anti-EGFR antibody (bottom) followed by stripping and reprobing with anti-phospho-EGFR (Tyr 845) antibody (top). B: 10 μM AG1478 abolished the hypotonic stress-induced phosphorylation of EGFR on Tyr 845. A6 cell monolayers were preincubated with vehicle or AG1478 for 60 min and then exposed to the hypotonic solution for the 10 min. This blot was initially blotted with anti-EGFR antibody (bottom) and then stripped and reprobed with anti-phospho-EGFR (Tyr 845) antibody (top). ISO, isotonic condition; HYPO, hypotonic condition.

Involvement of PI3-kinase in hypotonic stress-activated signaling pathway for \( I_{\text{Na}} \) and \( G_{\text{Na}} \). To study whether PI3-kinase is involved in the hypotonic stress-activated signaling pathway for \( I_{\text{Na}} \) and \( G_{\text{Na}} \), we applied LY294002, a specific inhibitor of PI3-kinase. LY294002 of 30 μM was applied to A6 cells 60 min before exposure to hypotonic stress, and LY294002 was present in the solution during the period for measurements of \( I_{\text{sc}} \) and \( G_{\text{t}} \). Figure 7A shows the time courses of changes in \( I_{\text{sc}} \), indicating that LY294002 drastically diminished the hypotonicity-induced \( I_{\text{sc}} \). Figure 7B shows the statistical results of \( I_{\text{Na}} \) at 120 min after exposure to hypotonic stress in the presence and absence of LY294002, clearly indicating the inhibitory action of LY294002 on \( I_{\text{Na}} \). The \( G_{\text{Na}} \) at 120 min after exposure to hypotonic stress was also diminished by treatment with LY294002 (Fig. 7C). These observations suggest that PI3-kinase would be involved in the hypotonicity-activated signal pathway for \( I_{\text{Na}} \) and \( G_{\text{Na}} \).

To confirm the involvement of PI3-kinase in the hypotonic stress-activated signaling pathway, we studied whether hypotonic stress affects the level of phosphorylated PKB that is phosphorylated by a PI3-kinase-dependent pathway (8, 33); namely, the phosphorylated level of PKB on both Thr 308 and Ser 473 can be used as an indicator of PI3-kinase activity. Figure 8A shows that hypotonic stress increased the phosphorylation level of PKB on both Thr 308 and Ser 473 and that LY294002 abolished the phosphorylation of PKB (Fig. 8B). These observations suggest that 1) the phosphorylation of PKB on Thr 308 and Ser 473 can be used as an indicator of PI3-kinase activity and 2) hypotonic stress activates PI3-kinase. Furthermore, we tried to clarify whether the hypotonicity-induced activation of PI3-kinase is mediated through RTK. Coapplication of AG1478 and AG1296 drastically diminished the hypotonicity-induced phosphorylation of PKB (Fig. 8C), suggesting that hypotonic stress activates PI3-kinase via activation of RTK. As described above, the hypotonicity-induced increases in \( I_{\text{Na}} \) and \( G_{\text{Na}} \) would be mediated through JNK/SAPK. Therefore, we studied whether JNK/SAPK participates in the hypotonicity-induced activation of PI3-kinase. JNK inhibitor II diminished the hypotonicity-induced phosphorylation of PKB (Fig. 8D), suggesting that hypotonic stress modulates PI3-kinase activity via activation of JNK/SAPK. On the other hand, inhibition of ERK1/2 by U-0126 did not affect the hypotonicity-induced phosphorylation of PKB (Fig. 8D), suggesting that the hypotonicity-activated RTK-ERK1/2 cascade is not involved in the activation of PI3-kinase by hypotonic stress.

Contribution of RTK to the early phase of \( I_{\text{Na}} \) transport induced by hypotonic stress. Rozansky et al. (41) have previously reported that the activation of \( I_{\text{Na}} \) transport in response to hypotonic stress could be divided into two phases: the early and late phases. In their report, they have shown that the early phase rapidly appears just after exposure to hypotonic stress.
independently of protein synthesis, whereas the late phase is the subsequent increase in \( I_{\text{Na}} \), appearing 30 min after exposure to hypotonic stress depending on synthesis of protein, possibly serum- and glucocorticoid-inducible kinase (SGK). Therefore, to clarify in which phase of the hypotonic action on \( Na^+ \) transport RTKs participates, we studied the effect of RTK inhibitors (coapplication of AG1478 and AG1296) on the early and late phases of the hypotonicity-stimulated \( I_{\text{Na}} \). Coapplication of AG1478 and AG1296 diminished the early phase appearing just after application of hypotonic stress (Fig. 9). On the other hand, an inhibitor of protein synthesis, cycloheximide (20 \( \mu \text{M} \)), diminished the late phase without any inhibitory effects on the early phase of \( I_{\text{Na}} \)-stimulated hypotonic stress. The cycloheximide-sensitive phase appeared \( \sim 2–3 \text{ h} \) after exposure to hypotonic stress in our experimental setup. Next, we examined the effect of cycloheximide in the presence of RTK inhibitors on the \( I_{\text{Na}} \) stimulated by hypotonic stress. Even in the presence of RTK inhibitors, cycloheximide affected the late phase \( \sim 2–3 \text{ h} \) after exposure to hypotonic stress, almost identical to that in the absence of RTK inhibitors (Fig. 9). These observations indicate that the hypotonicity-induced early phase is mediated through RTK activation independently of stimulation of protein synthesis. Our previous report indicated that hypotonic stress stimulated translocation of ENaC into the apical membrane (39). Taken together, these observations suggest that the hypotonic stress increases \( I_{\text{Na}} \) and \( G_{\text{Na}} \) through two processes: 1) the RTK-mediated early phase via translocation of presynthesized ENaC before hypotonic stress and 2) the RTK-independent late phase via new ENaC- and/or SGK-synthesizing processes.

**DISCUSSION**

**RTK-mediated mechanism of acute osmoregulation of \( Na^+ \) reabsorption.** Since Ussing and his colleagues originally proposed the relationship between hypotonic stress and active \( Na^+ \) transport in frog skin (25, 49), many studies have been performed to elucidate the precise mechanism by which changes in osmolality regulate \( Na^+ \) transport in tight epithelia. However, a definitive mechanism remains to be defined, although much remarkable progress has been made. Our previous studies indicated both acute \( \sim 2 \text{ h} \) (37, 39) and chronic \( \sim 6 \text{ h} \) (36) osmoregulation of \( Na^+ \) transport in A6 cells. In the case of a chronic reduction of intracellular osmolality, the decrease in cytosolic \( Cl^- \) concentration stimulated \( \alpha\)-ENaC mRNA expression and \( Na^+ \) transport (36), whereas acute hyposmoregulation of \( Na^+ \) transport was carried out mainly via an increase in the number of conducting ENaCs in the apical membrane in a PTK-dependent manner (39). However,
we have no information on what type of PTK is involved in the acute hyposmoregulation of Na⁺ transport.

The present study suggests that EGFR is at least ligand independently activated by hypotonicity and mediates the stimulatory action of hypotonic stress on Na⁺/H⁺ transport by increasing the benzamil-sensitive Gᵢ in response to hypotonic stress, although Tong and Stockand (48) have reported that activation of EGFR by EGF inhibits ENaC activity (open probability). Their report (48) seems to be apparently contradictory to our results shown in the present report. However, this apparent contradiction would be due to the difference between targets of EGFR activation in their study and ours as follows. They indicate the inhibitory effects of EGFR activation on ENaC activity in Chinese hamster ovary cells coexpressing α₁, β₁, and γ-hENaC and EGF receptor HER1. On the other hand, we suggest a stimulatory effect of EGFR activation on Na⁺/H⁺ transport (not ENaC activity) through endogenous X. laevis ENaC in hypotonic stress in amphibian epithelial A6 cells without an overexpressing system. Their experimental conditions are quite different from ours. Furthermore, we consider that the stimulatory mechanism of Na⁺ transport in hypotonic stress is mainly dependent on translocation of preexisting ENaC from cytosolic store sites to the apical membrane (39) not by increasing ENaC activity (open probability). On the other hand, Markadieu et al. (27) have reported that EGF stimulates Na⁺ transport via activation of EGFR in A6 cells. This study supports our results that activation of EGFR stimulates Na⁺ transport.

Pretreatment with AG1478 or AG1296, respectively, reduced hypotonicity-stimulated Na⁺ transport to ~50 or 60% of control, while pretreatment with coapplication of these inhibitors reduced it to ~30% of control (Fig. 2). If EGFR and PDGFR independently contribute to stimulate the Na⁺ transport in response to hypotonic stress, the I_{Na} in the presence of AG1478 and AG1296 should be <30% of control (~10% of control). This observation suggests that EGFR and PDGFR would share a common signal pathway as their downstream signalings participating in the hypotonic stress-induced stimulation of Na⁺ transport. Furthermore, the other part of Na⁺ transport, which is insensitive to both AG1478 and AG1296, might depend on other types of RTK or RTK-independent pathways.

In several types of cells (17, 46), hypotonic stress increases protein tyrosine phosphorylation, and interestingly a study in cardiac myocytes (43) provides evidence that hypotonic stress immediately activates PTK and this activation is mimicked by chlorpromazine, WHICH is known to cause membrane deformation. This report (43) suggests that hypotonic stress may activate PTK by changing membrane tension/deformation and that the activated PTK may play an essential role in response to environmental changes. Furthermore, Rosette and Karin (42) have discovered a novel mechanism in the activation of cell surface receptors including EGFR in response to osmotic stress and UV irradiation, so-called transactivation of RTK. In their report (42), membrane deformation caused by osmotic stress may alter conformation of cell surface receptors, leading to their activation. One of major growth factor receptors affected by membrane stretch/deformation is EGFR. Conformational alteration of EGFR causes autophosphorylation of tyrosine residues that produces high-affinity binding sites for the src

**Fig. 7. Effects of PI3-kinase (PI3-kinase) inhibitor (LY294002) on hypotonic stress-stimulated Na⁺ transport.** A: 60 min before exposure to the hypotonic solution, A6 cell monolayers were preincubated with vehicle (DMSO, ○) or LY294002 (30 μM, ●), which was also present in the hypotonic solution. Benzamil (10 μM) was applied to the apical side at 120 min after exposure to hypotonicity (n = 4). B and C: effects of 30 μM LY294002 on the benzamil-sensitive I_{sc} (B) and Gᵢ (C) at 120 min after exposure to the hypotonic solution. LY294002 significantly diminished the hypotonic stress-stimulated benzamil-sensitive I_{sc} and Gᵢ (n = 4). *P < 0.05.
homology 2 (SH2) domain of adaptor proteins. EGFR kinase then recruits and phosphorylates various adaptors and signaling molecules that bind phosphotyrosine motifs to their SH2 domain and phospho-tyrosine binding (PTB) domain, and thereby EGFR kinase transduces the signal to its downstream signaling cascades including ERK1/2- and JNK-dependent pathways. Previous studies (18, 24, 45) have shown that EGFR kinase undergoes transactivation on membrane stretch/deformation without ligand binding in several types of cells. These studies support our results that hypotonic stress-induced membrane stretch/deformation causes transactivation of RTK including EGFR involved in stimulation of Na⁺/H⁺ transport. In A6 cells, hypotonic stress activates intracellular signal molecules such as ERK1/2, JNK/SAPK, and p38 MAPK through cell swelling-induced membrane tension (38). Similar to various types of cells, hypotonic stress in A6 cell monolayers causes initial cell swelling followed by regulatory volume decrease (RVD) to return cell volume toward the original volume (14, 15). The electron micrographs of hypotonicity-treated A6 cell monolayers (51) indicate that the basolateral, not apical, membrane appears to be tightly stretched due to the initial cell swelling. Taken together, it is speculated that physical stresses to the basolateral membrane caused by hypotonic stress alter the conformation of RTK (EGFR) existing in the basolateral membrane, thereby activating downstream signaling pathways of growth factors through activation of RTK.

**RK-JNK/SAPK cascade activated by hypotonic stress mediates hypotonic activation of Na⁺ reabsorption.** Since the mechanism activating the members of the MAPK family in response to hypotonic stress in A6 cells has not yet been clarified, it is noteworthy that hypotonic stress activated ERK1/2 and JNK/SAPK through RTK shown in the present study.

**Fig. 8. Effects of LY294002, AG1478/AG1296, and JNK inhibitor II on hypotonic stress-increased phosphorylation of PKB.**

A: hypotonic stress transiently increased the phosphorylation of PKB on both Thr 308 and Ser 473. B–D: hypotonic stress increased the phosphorylated levels of PKB on both Thr 308 and Ser 473. LY294002 (30 μM; B), coapplication of 10 μM AG1478 and 10 μM AG1296 (C), and 10 μM JNK inhibitor II (D), but not 2 μM U-0126 (D), diminished the hypotonic stress-stimulated phosphorylation of PKB on both Thr 308 and Ser 473. A6 cells were treated with LY294002, coapplication of AG1478 and AG1296, JNK inhibitor II, or U-0126 for 60 min and then exposed to the hypotonic solution. These blots (A–D) were initially probed with anti-phospho-PKB (Thr 308) antibody (top) and then stripped and reprobed with anti-phospho-PKB (Ser 473) antibody (middle). Finally, blots were stripped and reprobed with anti-PKB antibody (bottom) as a loading control.
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study (Fig. 4). However, it is still unclear how hypotonic stress activates p38 MAPK (see Fig. 10).

We further studied whether the MAPK family signaling pathways are involved in the hypotonic stress-induced increase in Na⁺ transport. The present study indicates that among three members of the MAPK family, only JNK/SAPK was required for the hypotonic stress-induced stimulation of Na⁺ transport (Fig. 5), suggesting that activation of the RTK-JNK/SAPK cascade caused by hypotonic stress plays an essential role in the hypotonic action on Na⁺ transport (see Fig. 10). To our knowledge, a relationship between JNK/SAPK and amiloride-sensitive Na⁺ channels has not been proposed in any types of cells. Therefore, this is a novel regulatory mechanism of Na⁺ transport through an RTK-JNK/SAPK cascade in hypotonic stress, although a report by Markadieu et al. (27) suggests that RTK acts on Na⁺ transport without any description of the RTK-JNK/SAPK cascade. In A6 cell monolayers, like insulin, EGF stimulates Na⁺ transport by increasing the activity of PI3-kinase and thereby generates phosphatidylinositol 3,4,5-trisphosphate (PIP₃) (4, 6, 27, 40). Among the products of PI3-kinase, PIP₃, and to a lesser extent PI(3,4,4)P₂, has been shown to increase Na⁺ transport (26, 47). The activation of PI3-kinase by EGF results from the recruitment of the SH2 domains of the p85 regulatory subunit of PI3-kinase to a phosphorylated tyrosine on the EGFR or on some associated adaptors, bringing the catalytic p110 subunit close to its substrates [PI(4,5)P₂], and allowing the production of PIP₃. Therefore, we examined a relationship between the RTK-JNK/SAPK cascade and the activity of PI3-kinase in hypotonic stress. As the activation of PI3-kinase results in PKB phosphorylation (8), the immunodetection of phosphorylated PKB on both Thr 308 and Ser 473 was used as an indicator of activity of PI3-kinase. Hypotonicity increased the level of phosphorylated PKB on both Thr 308 and Ser 473, and the activation of PKB by hypotonic stress was diminished by the inhibition of RTK or JNK/SAPK (Fig. 8). These results suggest a possibility that the RTK-JNK/SAPK cascade modulates PI3-kinase activity (see Fig. 10), although the mechanism of this modulation remains to be defined. It might appear surprising that JNK/SAPK may modulate PI3-kinase activity, but an interaction between PI3-kinase and another member of the MAPK family, ERK1/2, has been reported in epithelia chronically exposed to aldosterone.

Fig. 9. Effects of protein synthesis inhibition on the hypotonic stress-stimulated Iₛₑ in the absence and presence of coapplication of AG1478 and AG1296. A: in the absence and presence of coapplication of 10 μM AG1478 and 10 μM AG1296, A6 cell monolayers were incubated with vehicle (0.1% DMSO) or 20 μM cycloheximide for 30 min and then exposed to the hypotonic solution. ○, AG1478/AG1296 (−) and cycloheximide (−); □, AG1478/AG1296 (−) and cycloheximide (+); ■, AG1478/AG1296 (+) and cycloheximide (−); ●, AG1478/AG1296 (+) and cycloheximide (+). The inhibitory action of coapplication of AG1478/AG1296 was observed in hypotonic stress-stimulated Iₛₑ for all the time periods under hypotonic condition (compare closed symbols with open symbols). On the other hand, cycloheximide diminished the Iₛₑ (−180 min after application of hypotonicity (compare open squares with open circles). AG, coapplication of AG1478 and AG1296 (n = 4–5). B: RTK-dependent and cycloheximide-sensitive components of hypotonicity-stimulated Iₛₑ. The RTK-dependent Iₛₑ (○) appeared just after exposure to hypotonic stress, while the cycloheximide (CHX)-sensitive Iₛₑ (■) was observed −180 min after application of hypotonic stress (n = 4–5).

Fig. 10. Hypothesized scheme of the acute osmoregulation of Na⁺ reabsorption through an RTK-JNK/SAPK cascade. (1), Hypotonic stress activates RTKs; (2), RTKs increase the activity of ERK1/2 and JNK/SAPK; (3), JNK/SAPK, but not ERK1/2, is involved in the hypotonic stress-induced translocation of epithelial Na⁺ channel (ENaC) into the apical membrane through the modulation of PI3-kinase activity. (+) signifies a stimulatory effect; circled P, phosphorylated; PIP₃, phosphatidylinositol 3,4,5-trisphosphate.
(19, 23) and in epithelia treated with EGF (27). The activation of ERK1/2 by EGF shows inhibitory effects on both Na⁺ transport and PI3-kinase activity (27), whereas in the case of hypotonic stress the activated ERK1/2 did not show any inhibitory effects on Na⁺ transport or PI3-kinase activity (Figs. 5 and 8D). These observations lead us to consider that the ligand-independent “transient” activation of EGFR by hypotonic stress might be different from ligand-dependent “sustained” activation of EGFR by EGF with regard to Na⁺ transport and PI3-kinase activity. Furthermore, in addition to the EGFR-ERK1/2 pathway, hypotonic stress activates various signaling pathways affecting Na⁺ transport and PI3-kinase activity differently from activation of EGFR by EGF. Clarification of the detailed mechanisms of the RTK-JNK/SAPK cascade stimulating Na⁺ transport would be a next challenge.

Requirement of RTK-JNK/SAPK cascade in nongenomic action of hypotonic stress on Na⁺ reabsorption. The induction of Na⁺ transport by hypotonic stress can be divided into two phases (41). One is the protein synthesis-independent, early phase (~2 h) and the other is the protein (possibly SGK) synthesis-dependent, late phase (~3 h). Transactivation of RTK by hypotonic stress acts only on the early phase but not on the late phase (Fig. 9). Since long-term treatment with JNK inhibitor II (~2 h) was slightly toxic to A6 cell monolayers and gradually increased Gᵣ to levels that did not allow us to have trustworthy electrophysiological recordings (i.e., the monolayers became leaky), it was impossible to measure Iₛ and Gᵣ for a long duration in the presence of JNK inhibitor II for clarification of JNK action on the late phase. Moreover, the hypotonic induction of SGK did not require the activity of RTK or JNK/SAPK, and inhibition of RTK did not affect the increase in Na⁺ transport by overnight reduction in extracellular osmolality (Taruno A, unpublished observations). The data strongly suggest that, like aldosterone (7, 10, 52), hypotonic stress has both genomic and nongenomic effects on Na⁺ transport and that the RTK-JNK/SAPK cascade mediates nongenomic stimulation constituting the early phase of the hypotonic action on Na⁺ transport.

Amount of benzamil-sensitive Iₛ. The benzamil-sensitive Iₛ shown in the present study was much smaller than that observed in A6 cells by others (4, 54). Wills and colleagues (54) have observed the hypotonic action on the Iₛ in A6 cells with a higher amount of Iₛ than in our study. Therefore, the hypotonic effect on Na⁺ transport shown in the present study would not be due to the initial low value of Iₛ, and the involvement of RTK in this process might not depend on the low initial value of Iₛ. The low value of Iₛ observed in the present study would be due to the culture condition, including the culture insert and the medium, although these should be further investigated in detail.

Conclusion. We indicate the possible role of the RTK-JNK/SAPK signaling pathway in the acute osmoregulation of Na⁺ reabsorption across A6 cell monolayers mainly carried out through ENaC transllocation into the apical membrane via a protein-synthesis-independent pathway. Furthermore, we indicate that the action of the RTK-JNK/SAPK cascade on Na⁺ transport would be mediated through PI3-kinase, although it is still unclear how the RTK-JNK/SAPK cascade modulates the activity of PI3-kinase or whether the RTK-JNK/SAPK cascade directly regulates ENaC activity. Further experiments are required to clarify these points (see Fig. 10).

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