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Aldosterone-induced modification of osmoregulated ENaC trafficking

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Abstract

Aldosterone and osmotic stress are well known to regulate the epithelial Na^+ channel (ENaC)-mediated Na^+ transport in renal epithelial cells. However, we have no information on how aldosterone and osmotic stress interact on stimulation of ENaC-mediated Na^+ transport in renal epithelium. In the present report, we studied how application of aldosterone (1 μM for 1 day) modifies the action of hypotonic stress on the ENaC-mediated Na^+ transport in renal A6 epithelial cells by measuring the benzamil (a specific inhibitor for ENaC)-sensitive short-circuit current. The present study suggests that: (1) most ENaCs in cells without aldosterone treatment are translocated to Golgi apparatus, (2) major parts of aldosterone-generated ENaCs are located at the endoplasmic reticulum, (3) aldosterone diminishes the endocytosis rate of ENaCs from the apical membrane without any significant changes in the insertion rate of ENaCs into the apical membrane, and (4) application of sucrose after hypotonic stress stimulates the endocytosis of ENaCs, and elongates the functional life time of ENaCs by enhancing recycle of ENaCs into the endoplasmic reticulum in a retrograde manner.

Keywords: Epithelial ion transport; Renal epithelium; Short-circuit current; ENaC; Aldosterone; Hypotonic stress

Aldosterone controls body fluid content and blood pressure [1–6] by regulating the epithelial Na^+ channel (ENaC)-mediated transepithelial Na^+ absorption in renal epithelial cells [7–10]. The ENaC-mediated transepithelial Na^+ absorption is carried out by two steps; (1) the entry step of Na^+ across the apical membrane via ENaC, and (2) the extrusion step of Na^+ across the basolateral membrane mediated by the Na^+, K^+-pump [11]. ENaCs participating in the entry step of Na^+ across the apical membrane in Na^+-absorbing epithelial cells are the rate-limiting step in the transepithelial Na^+ absorption under most circumstances [11]. Therefore, regulation of ENaCs is the principle mechanism by which the transepithelial Na^+ absorption is altered. Aldosterone is well known to stimulate the transepithelial Na^+ absorption by elevating ENaC gene expression [1,3,12]. Although the stimulatory action of aldosterone on ENaC has been studied in detail, the mechanism is still unclear and under investigation. Hypotonic stress is well known to stimulate translocation of ENaCs to the apical membrane, elevating the transepithelial Na^+ absorption [13,14]. However, we have no information on how aldosterone controls the location of ENaC regulated by osmotic stress. In the present study, we investigated the aldosterone action on the osmotic stress-regulated translocation of ENaC. The present study reports for the first time here that aldosterone modifies the osmotic stress-induced regulation of ENaC trafficking.

Materials and methods

Solvents of chemicals. Aldosterone was dissolved in ethanol. Benzamil was dissolved in dimethyl sulfoxide (DMSO). The final concentrations of ethanol and DMSO used in the present study were 0.1%. Ethanol or DMSO alone at the concentration had no effects on the short-circuit current (Isc).

Cell culture. We purchased A6 cells at the 68th passage from American Type Culture Collection (Manassas, VA, USA), and cultured A6 cells of

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passage 75–84 on plastic flasks in an NCTC-109 culture medium modified for amphibian cells containing 10% fetal bovine serum, streptomycin, and penicillin in a humidified incubator at 27 °C with 1.0% CO_2 in air similar to our previous study [15–18]. We seeded A6 cells onto polycarbonate porous membranes attached to the bottom of 6.5 mm Tissue culture-treated Transwell filter plastic cups (Costar Corporation, Cambridge, MA, USA) at density of 5×10^4 cells/well for 14–16 days.

Washing solution. We washed A6 cells using a 120 mM NaCl solution containing 120 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM *N*–2–hydroxy–ethylpiperazine–*N*'–2–ethanesulfonic acid (Hepes). We adjusted the pH of the solution to 7.4 with NaOH.

Application of hypotonic stress. We applied hypotonic stress by incubating A6 cells in a hypotonic solution containing 60 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM Hepes for the apical and basolateral solutions. We adjusted the pH of the solution to 7.4 with NaOH.

Application of sucrose for recovery of the osmolality. We applied 120 mM sucrose to increase the osmolality to that just before application of hypotonic stress to the apical and basolateral solutions. The solution contained 120 mM sucrose, 60 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM Hepes. We adjusted the pH of the solution to 7.4 with NaOH.

Measurements of Isc. We rinsed (washed) the A6 cell monolayers grown on polycarbonate porous membranes with the same solution as the experimental solution, and transferred the A6 cell to a modified Ussing chamber (Jim's Instrument, Iowa City, IA, USA) that was designed for holding the cup with polycarbonate porous membrane [13,14,19,20]. We continuously measured a transepithelial electrical–potential-difference (PD) across the A6 cell monolayer using a high-impedance millivoltmeter that could function as a voltage clamp with automatic fluid resistance compensation (VCC-600, Physiologic Instrument, San Diego, CA, USA) under the condition that the apical and basolateral solutions in Ussing chamber were electrically connected to with a pair of calomel electrodes immersed in a saturated KCl solution and bridged to Ussing chamber by a pair of polyethylene tubes filled with a solution of 2% (weight/volume (w/v)) agarose containing 2 M KCl. We applied a pulse of 1 μA constant current for 0.5 s to the A6 cell monolayer under an open-circuit condition every 10 s, calculating the transepithelial conductance (Gt) from the change in the PD (ΔPD) caused by a 1 μA constant current pulse using Ohm's law (Gt = 1 μA/ΔPD mV) [21]. Using the measured Gt and PD under an open-circuit condition, we estimated the Isc (the equivalent current) by calculating the product of Gt and PD ($Isc = Gt \times PD$) [21]. A positive current represents a net flow of cation from the apical to the basolateral solutions; i.e., the transepithelial Na^+ absorption is represented as a positive current (Isc) in the present study. Benzamil is a specific blocker of ENaC, and benzamil of 10 μM completely blocks the activity of ENaC [22–24]. Therefore, the benzamil-sensitive Isc was used as an indicator of the transepithelial Na^+ absorption mediated through ENaC. The bathing solution was stirred with 21% O_2/79% N_2. We show the Isc normalized to that at 4 h after application of hypotonic stress.

Pretreatment with aldosterone in culture medium. When we pretreated A6 cells with aldosterone, we added aldosterone of 1 μM into the culture media for 24 h before we moved A6 cells to the Ussing chamber for measurements of Isc. When we measured Isc, we washed out aldosterone with the same solution as the experimental solution in the Ussing chamber. Aldosterone was not present in a solution for measurement of Isc in the Ussing chamber.

Estimation of the rates of insertion and endocytosis of ENaC into and from the apical membrane: we estimated the rates of insertion (α) and endocytosis (β) of ENaC into and from the apical membrane, respectively as follows:

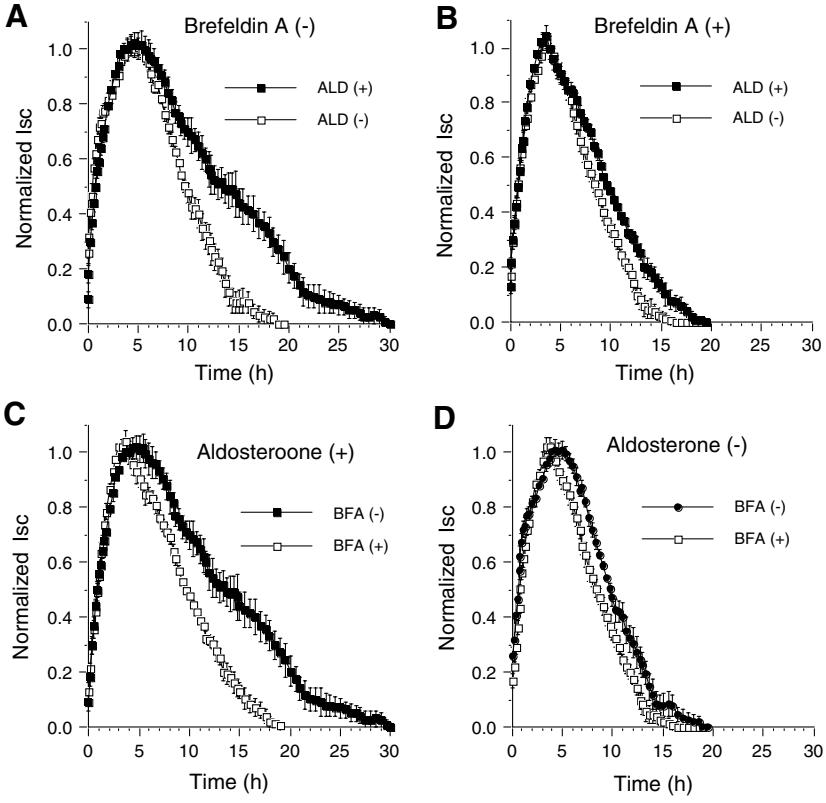

Fig. 1. Effects of aldosterone and brefeldin A on the hypotonic stress-induced I_{Na}. (A) Effects of aldosterone on I_{Na}. (B) Effects of aldosterone on I_{Na} in brefeldin A-treated cells. Brefeldin A was added into the apical and basolateral solutions at 3 h after application of hypotonic stress. (C) Effects of brefeldin A on I_{Na} in aldosterone-treated cells. Brefeldin A made the declining phase faster. (D) Effects of brefeldin A on I_{Na} in aldosterone-untreated cells. $n = 5$.

$$d[ENaC]/dt = \alpha[A] - \beta[ENaC] \quad (1)$$

$$d[A]/dt = -\alpha[A] \quad (2)$$

where [ENaC] is the amount of ENaCs at the apical membrane, [A] is the amount of ENaCs in the intracellular pool (the endoplasmic reticulum and Golgi apparatus), and t is the time after application of hypotonic stress. The following Eq. (3) is induced from Eq. (2):

$$[A]=[A_o]\exp(-\alpha t) \quad (3)$$

where $[A_o]$ is the amount of ENaCs in the intracellular pool (the endoplasmic reticulum and Golgi apparatus) just before application of hypotonic stress. The following Eq. (4) is induced from Eqs. (1) and (3):

$$d[ENaC]/dt=\alpha\,[A_o]\exp(-\alpha t) - \beta\,[ENaC] \quad (4)$$

We estimated the rates of insertion (α) and endocytosis (β) of ENaC into and from the apical membrane fitting Eq. (4) to the observed Isc.

Chemicals. We obtained benzamil from Sigma (St. Louis, MO, USA), and NCTC-109 medium and fetal bovine serum from GIBCO (Grand Island, NY, USA). We purchased all other chemicals not listed above from Sigma (St. Louis, MO , USA).

Temperature. All experiments were performed at 22–23 °C.

Data presentation and statistics. Results are expressed as the mean ± standard error (SE). Statistical significance was determined by Student's t-test or ANOVA, and $p < 0.05$ was considered significant.

Results and discussion

We measured the Isc in the absence of serum or any amino acids. Irrespective of aldosterone treatment (1 μM for 24 h), the Isc increased just after application of hypotonic stress at time 0 h (Fig. 1A) as previously reported [25–28]. The Isc reached its peak value around 4 h after application of hypotonic stress, declining toward 0 level with time in A6 cells treated with and without aldosterone (Fig. 1A). In the presence of 10 μM benzamil (a specific blocker of ENaC), we did not observe the increase in Isc (data not shown), suggesting that the hypotonic stress-induced increase in Isc is due to the ENaC-mediated trans-epithelial Na^+ transport (I_{Na}). Therefore, we recognized that the hypotonic stress-induced Isc is I_{Na}. Hereby, in the present study we call the hypotonic stress-induced Isc I_{Na}. The hypotonic stress-induced rising phase in I_{Na} was not significantly affected by aldosterone treatment (Fig. 1A); i.e., the time course in the hypotonic stress-induced rising phase in I_{Na} observed in aldosterone-treated cells was identical to that in aldosterone-untreated cells. In A6 cells without aldosterone treatment, the I_{Na} reached 0 level around 20 h after application of hypotonic stress (open symbols in Fig. 1A). On the other hand, in A6 cells treated with aldosterone, the I_{Na} was still observed around 20 h after application of hypotonic stress, reaching 0 level around 30 h after application of hypotonic stress (closed symbols in Fig. 1A). These observations indicate that aldosterone makes the declining phase of Isc slower with no effects on the rising phase, and that aldosterone keeps

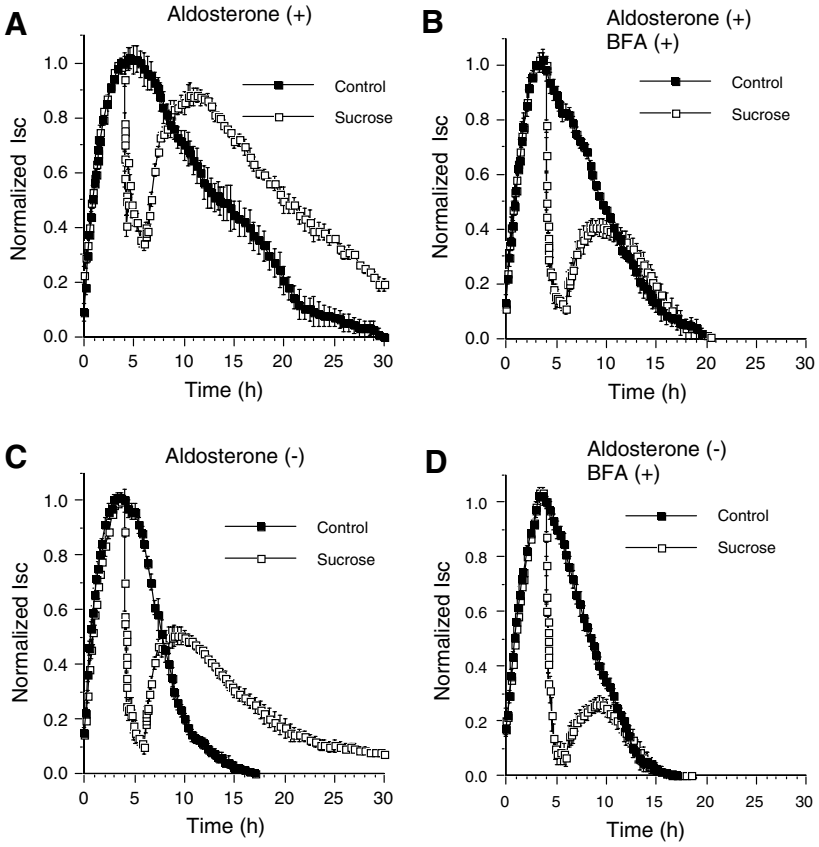

Fig. 2. Effects of sucrose and brefeldin A on the hypotonic stress-induced I_{Na} in aldosterone-treated and untreated cells. (A) Effects of aldosterone on I_{Na}. (B) Effects of aldosterone on I_{Na} in the presence of brefeldin A. (C) Effects of brefeldin A on I_{Na} in aldosterone-treated cells. Brefeldin A made the declining phase faster. (D) Effects of brefeldin A on I_{Na} in aldosterone-untreated cells. $n = 5$.

ENaC at some intracellular places, resulting in a slow kinetic of ENaC trafficking.

Next, we studied how brefeldin A (5 μg/ml, an inhibitor of protein trafficking from endoplasmic reticulum to Golgi apparatus [29–31]) modifies the action of aldosterone on the I_{Na}. Brefeldin A was added into the apical and basolateral solutions 3 h after application of hypotonic stress (Fig. 1B). The aldosterone action on the declining phase was diminished by the treatment with brefeldin A (compare Fig. 1B with A). To clearly show the effect of brefeldin A on I_{Na} in A6 cells treated with and without aldosterone, we again plotted the data (Fig. 1C and D). In aldosterone-treated A6 cells, brefeldin A shifted the declining phase leftward (Fig. 1C). This shift was much larger in aldosterone-treated cells (Fig. 1C) than that in cells without aldosterone treatment (Fig. 1D). Taken together, these observations suggest that the elongating action of aldosterone on the declining phase of I_{Na} shown in Fig. 1A is mediated via translocation of ENaC from the endoplasmic reticulum to Golgi apparatus due to a large number of aldosterone-generated of ENaCs at the endoplasmic reticulum. On the other hand, brefeldin A had little effects on I_{Na} in A6 cells without aldosterone treatment as shown in Fig. 1D, suggesting that in cells without aldosterone treatment little ENaC proteins are located at the endoplasmic reticulum, but most ENaC proteins are translocated to Golgi apparatus.

We next studied the effect of sucrose on the hypotonic stress-stimulated I_{Na} in aldosterone-treated cells. In aldosterone-treated A6 cells, the I_{Na} was rapidly diminished by bilateral application of sucrose (120 mM) at 4 h after cells were exposed to hypotonic stress (open symbols in Fig. 2A). The I_{Na} was recovered by removal of sucrose which was applied for 2 h between 4 and 6 h after application of hypotonic stress (open symbols in Fig. 2A). Interestingly, the recovered level of I_{Na} (open symbols in Fig. 2A) was higher than the I_{Na} without application of sucrose (closed symbols in Fig. 2A), and the total amount of Na^+ transport during the experimental period was larger in cells recovered from application of sucrose (open symbols in Fig. 2A) than that in cells without application of sucrose (closed symbols in Fig. 2A), namely the total life time of ENaCs would be elongated by temporary application of sucrose. The higher level of I_{Na} recovered by removal of sucrose and the increase in total amount of Na^+ transport was abolished by brefeldin A treatment

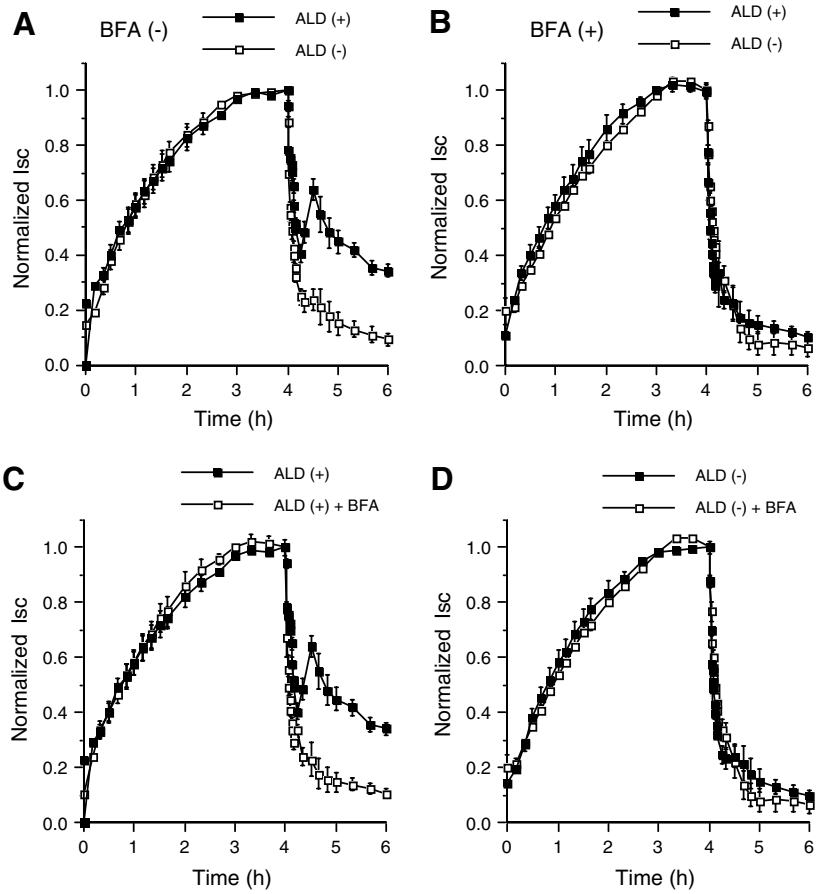

Fig. 3. Effects of aldosterone and brefeldin A on the sucrose-induced diminution of I_{Na}. (A) Effects of aldosterone on I_{Na}. The I_{Na} decreased in cells treated without aldosterone. On the other hand, in aldosterone-treated cells, the I_{Na} showed a transient increase following the sucrose-induced decrease in I_{Na}. (B) Effects of aldosterone on I_{Na} in the brefeldin A-treated cells. (C) Effects of brefeldin A on I_{Na} in aldosterone-treated cells. Brefeldin A abolished the transient increase in I_{Na}. (D) Effects of brefeldin A on I_{Na} in aldosterone-untreated cells. $n = 5$.

(Fig. 2B). These phenomena were also observed in A6 cells without aldosterone treatment (Fig. 2C and D). The level of I_{Na} at 2 h after bilateral application of sucrose was higher in aldosterone-treated cells (approximately 0.3; open symbol in Fig. 2A) than that in aldosterone-untreated cells (approximately 0.1; open symbol in Fig. 2C). This difference disappeared in brefeldin A-treated cells (open symbols in Fig. 2B and D). These observations suggest that application of sucrose enhances the endocytosis of ENaCs from the apical membrane into the endoplasmic reticulum irrespective of aldosterone treatment, elongating the total lifetime of ENaCs.

We found another action of aldosterone on I_{Na} at the sucrose-induced diminishing phase of I_{Na}. As described above, addition of sucrose rapidly diminished the I_{Na} (closed and open symbols in Fig. 3A). However, in A6 cells treated with aldosterone, the I_{Na} was recovered after application of sucrose (closed symbols in Fig. 3A). On the other hand, this recovery in I_{Na} was not observed in aldosterone-untreated cells (open symbols in Fig. 3A). This aldosterone-induced recovery in I_{Na} was almost completely diminished by brefeldin A (closed symbols in Fig. 3B). Fig. 3C and D clearly show the effect of brefeldin A on I_{Na}; brefeldin A almost completely diminished the recovery of I_{Na} in aldosterone-treated cells (Fig. 3C), but did not show any significant effects on I_{Na} in aldosterone-untreated cells (Fig. 3D). These observations suggest that some parts of ENaC proteins in aldosterone-treated cells are translocated to the apical membrane from the endoplasmic reticulum even in the presence of sucrose, but not in aldosterone-untreated cells. A simple explanation why the recovery of I_{Na} appears only in aldosterone-treated A6 cells is as follows: in aldosterone-treated cells, (1) many ENaCs are located in endoplasmic reticulum, (2) at application of sucrose many ENaCs are reversible translocated into endoplasmic reticulum in a retrograde pathway [30,32], (3) these translocated ENaCs into the endoplasmic reticulum in a retrograde manner push the aldosterone-generated ENaCs out of the endoplasmic reticulum into Golgi apparatus due

Table 1
The rates of insertion and endocytosis of ENaC into and from the apical membrane

h^{-1}	Insertion rate (α)	Endocytosis rate (β)
Aldosterone (−)	0.314 ± 0.016	0.219 ± 0.015
Aldosterone (+)	$0.325 \pm 0.020^{N.S.}$	0.131 ± 0.016^{a}

N.S., not significantly different between aldosterone (−) and (+), $n = 5$.
[a] Significantly different between aldosterone (−) and (+) at $p < 0.005$, $n = 5$.

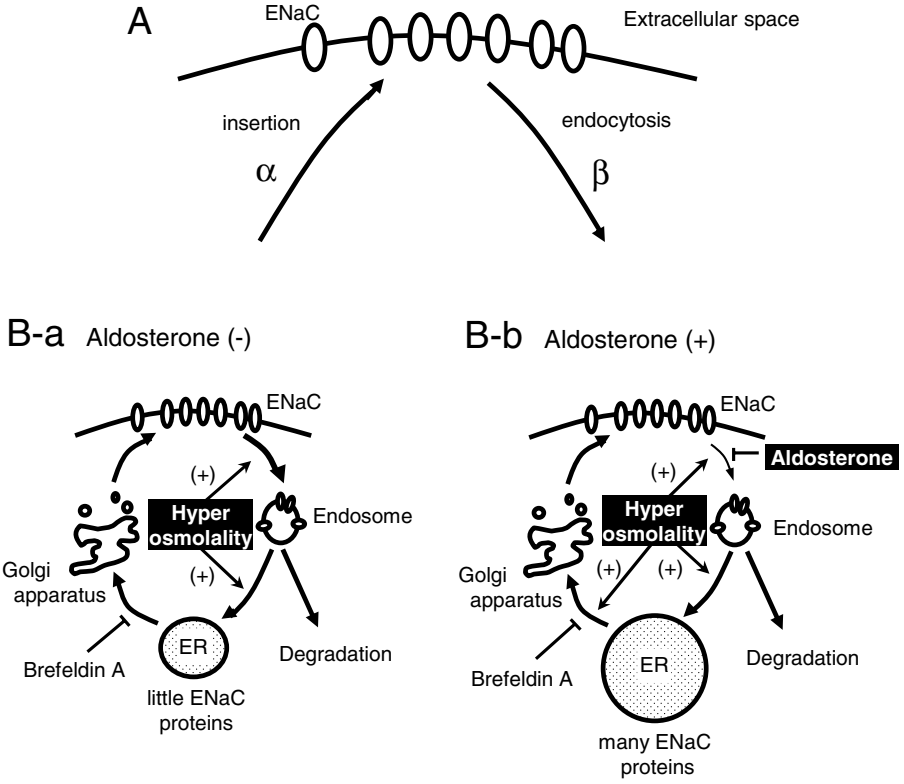

Fig. 4. A model of the intracellular trafficking of ENaCs. (A) A model of insertion and endocytosis of ENaCs into and from the apical membrane. α and β are, respectively, the rates of insertion and endocytosis of ENaCs. (B) Models of the intracellular trafficking of ENaCs in cells treated without (a) and with (b) aldosterone. Aldosterone decreases the endocytotic rate, and increases the number of ENaCs localized at the endoplasmic reticulum (b). An increase in osmolality enhances the endocytosis of ENaCs from the apical membrane, leading to translocation of ENaCs into the endoplasmic reticulum in a retrograde manner. The translocated ENaCs into the endoplasmic reticulum in a retrograde manner push aldosterone-generated ENaCs into Golgi apparatus out of the endoplasmic reticulum.

to a large number of ENaCs located at the endoplasmic reticulum. On the other hand, in cells treated without aldosterone, a significant number of ENaCs are not translocated to Golgi apparatus, since only a small number of ENaCs stay at the endoplasmic reticulum even if sucrose translocates ENaCs to the endoplasmic reticulum in a retrograde manner. We also further tried to determine the rates of insertion (α) and endocytosis (β) of ENaCs into and from the apical membrane based on a trafficking model of ENaCs shown in Fig. 4A. As shown in Table 1, aldosterone had no significant effects on the rate of insertion (α). This supports that the rising phase was not affected by aldosterone, since this phase would be mainly due to the insertion of ENaCs into the apical membrane. On the other hand, aldosterone decreased the rate of endocytosis (β) (Table 1). This supports that aldosterone made the declining phase slow, since this phase would be mainly due to the endocytosis of ENaCs from the apical membrane into the intracellular space.

Based on the observations shown in the present study, we propose a model on the intracellular trafficking of ENaCs (Fig. 4B-a and b); (1) most ENaC proteins in cells without aldosterone treatment are translocated to Golgi apparatus, but do not stay at the endoplasmic reticulum (Fig. 4B-a), (2) major parts of ENaC proteins generated by aldosterone treatment are located at the endoplasmic reticulum (Fig. 4B-b), (3) aldosterone makes the endocytotic rate of ENaCs slow (Fig. 4B-b), (4) application of sucrose after hypotonic stress stimulates the endocytotic trafficking of ENaCs into the endoplasmic reticulum, elongating the functional life time of ENaCs by enhancing recycle of ENaCs from the endosome to the endoplasmic reticulum in a retrograde manner (Fig. 4B-a and b), and (5) the sucrose-induced translocated ENaCs in a retrograde manner push ENaCs out of the endoplasmic reticulum into Golgi apparatus in aldosterone-treated cells, in which many ENaCs are located at the endoplasmic reticulum.

Acknowledgments

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