Analysis of Blocker-labeled Channels Reveals the Dependence of Recycling Rates of ENaC on the Total Amount of Recycled Channels

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Key Words

Epithelial ion transport • Ion channel • ENaC • Trafficking • Recycling

Abstract

Trafficking is one of the primary mechanisms of epithelial Na⁺ channel (ENaC) regulation. Although it is known that ENaCs are recycled between the apical membrane and the intracellular channel pool, it has been difficult to investigate the recycling of ENaCs; especially endogenously expressed ENaCs. The aim of the present study is to investigate if the recycling rates of ENaCs depend on the total amount of recycled ENaCs. To accomplish this point, we established a novel method to estimate the total amount of recycled ENaCs and the ENaC recycling rates by using a specific blocker (benzamil) of ENaC with a high-affinity for functional label of the channels in recycling. Applying this method, we studied if a decrease in the total amount of ENaCs caused by brefeldin A (5 µg/mL, 1 h) affects respectively the rates of insertion and endocytosis of ENaCs to and from the apical membrane in monolayers of renal epithelial A6 cells. Our observations indicate that: 1) both insertion and

endocytosis rates of ENaC increase when the total amount of ENaCs decreases, 2) the increase in the insertion rate is larger than that in the endocytosis rate, and 3) this larger increase in the insertion rate than the endocytosis rate caused by the decrease in the total amount of ENaCs plays an important role in preventing Na⁺ transport from drastically diminishing due to a decrease in the total amount of ENaCs. The newly established analysis of blocker-labeled ENaCs in the present study provides a useful tool to investigate the recycling of endogenously expressed ENaCs.

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Introduction

It is well known that epithelial Na⁺ channel (ENaC) contributes to regulation of body fluid content and blood pressure by mediating Na⁺ reabsorption in the kidney [1-

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5]. In the distal nephron (the cortical collecting duct) of the kidney, ENaCs are located in the apical membrane and mediate transepithelial Na^+ reabsorption in cooperation with the Na^+, K^+ -pump located in the basolateral membrane. In general, the Na^+ entry step through ENaCs, but not the Na^+ extrusion step through the Na^+, K^+ -pump, is considered as the rate-limiting step in the transepithelial Na^+ transport. Thus, to identify regulatory mechanisms of ENaC activity is crucial for a better understanding of regulation of transepithelial Na^+ transport. ENaC-mediated Na^+ transport in renal epithelium mainly depends on the number of ENaCs in the apical surface but not on the activity of individual ENaC (i.e., open probability) [6–10]. Since ENaCs are recycled between the apical membrane and the intracellular channel pool, the amount of apical surface ENaCs (S_{ENaC}) depends on the total amount of recycled ENaCs (T_{ENaC}) and the balance between the rates of insertion and endocytosis [8]. However, we have no information on the relationship between T_{ENaC} and the recycling rates of ENaCs. In the present study, to estimate T_{ENaC} and the recycling rates, we labeled recycled ENaCs with temporal application of a high-affinity, specific blocker of ENaC, benzamil, and detected the benzamil labeling-sensitive component of Na^+ current as Na^+ transport achieved by recycled ENaCs. With this novel method, we investigated the interaction between T_{ENaC} and the rates of ENaC recycling. The present study reports for the first time that the recycling rates of ENaC depend on T_{ENaC} .

Materials and Methods

Solvents of chemicals

Benzamil and amiloride were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO used in the present study was 0.1%. DMSO alone at the concentration had no effects on the short-circuit current (I_{sc}).

Cell culture

Renal epithelial A6 cells derived from *Xenopus laevis* were purchased from American Type Culture Collection (Rockville, MD, U.S.A.) at passage 68. A6 cells (passage 76–84) were grown on plastic flasks in a humidified incubator in a culture medium containing 75% (v/v) NCTC-109, 15% (v/v) distilled water and 10% (v/v) fetal bovine serum at 27 °C with 1.0% CO_2 in air as described in our previous studies [11–16]. Cells were seeded onto tissue culture-treated Transwell filter cups with polycarbonate porous membranes (Costar Corporation, Cambridge, MA, USA) for measurements of I_{sc} at a density of 5×10^4 cells/well and were cultured for 11–15 days.

Measurements of I_{sc}

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, U.S.A.) that was designed for holding the cup with polycarbonate porous membrane [17–19]. 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, U.S.A.) 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 constant current of 1 μA 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 ($Gt = 1 \mu\text{A} / \Delta\text{PD mV}$) [18]. Using the measured Gt and PD under an open-circuit condition, we estimated the I_{sc} (the equivalent current) by calculating the product of Gt and PD ($I_{\text{sc}} = Gt \times \text{PD}$) [18]. 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 (I_{sc}) in the present study. The bathing solution was stirred with 21% O_2 / 79% N_2 .

Application of hypotonic stress

We applied hypotonic stress to A6 cells by changing a bathing solution from an isotonic solution to a hypotonic solution for the apical and basolateral solutions. The isotonic solution (255 mOsm/kg H_2O) contained 120 mM NaCl, 3.5 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 5 mM glucose. The hypotonic solution (135 mOsm/kg H_2O) contained 60 mM NaCl, 3.5 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, and 5 mM glucose. We adjusted the pH of the solutions to 7.4 with NaOH. To prevent ENaCs located in the trans-Golgi network from being translocated into the apical membrane during the hypotonic stress, we supplemented the hypotonic solution with brefeldin A (BFA, 5 $\mu\text{g}/\text{ml}$) in some experiments.

Chemicals

We obtained benzamil and amiloride from Sigma (St. Louis, MO, U.S.A.), and NCTC-109 medium and fetal bovine serum from GIBCO (Grand Island, NY, U.S.A.). We purchased all other chemicals from Sigma (St. Louis, MO, U.S.A.).

Temperature

All experiments were performed at 22–23 °C.

Data presentation and statistics

Results are expressed as the mean $\pm$ standard error (S.E.). Statistical significance was determined by Student's *t*-test or ANOVA appropriately, and $p < 0.05$ was considered significant.

Results and Discussion

Blocker labeling of recycled ENaCs: Amiloride and its analog, benzamil, are two of the most famous blockers of ENaC [20]. First, we investigated the dose-dependent effects of these blockers on I_{SC} in aldosterone-pretreated A6 cells. Aldosterone pretreatment was only used for experiments (Fig. 1A) to facilitate precise analysis of inhibitory effects of benzamil and amiloride on I_{SC} under a condition with enhanced expression of ENaC, and not in further experiments in the present study. As shown in Fig. 1A, these blockers diminished I_{SC} in a dose-dependent manner and IC_{50} of benzamil and amiloride was 4.9 nM and 36.9 nM, respectively. These values are consistent with those obtained by single channel recording techniques [21–23]. Based on these observations, we considered the benzamil (1 μ M)- and amiloride (10 μ M)-sensitive I_{SC} as ENaC-mediated Na^+ current (I_{Na}). The difference in blocking potencies of amiloride and benzamil is known due to the remarkable difference in the rate of dissociation from ENaC, but not the rate of association to ENaC. Since benzamil has a much lower dissociation rate than that of amiloride, benzamil seems to irreversibly bind to ENaC [8, 22]. Therefore, it is expected that benzamil can functionally label ENaCs located at the apical membrane; that is, benzamil-labeled ENaCs with no activity have no contribution to I_{SC} , even if benzamil-labeled ENaCs are recycled to the apical surface. Hypotonic stress stimulates ENaC translocation to the apical membrane from the cytosolic space [6, 7]. ENaCs in cytosolic space is not labeled by apically applied benzamil, therefore these ENaCs are detected as an increase in I_{SC} when ENaCs are translocated into the apical membrane. To investigate the usability of benzamil for ENaC labeling located in the apical membrane, benzamil of 1 μ M or amiloride of 10 μ M was apically applied to A6 cells for 120 s (2 min) under an isotonic condition (Fig. 1B-a). Then, the apical solution was washed extensively (5-fold volume exchange) to remove the blockers completely and changed to the hypotonic solution without the blockers (Fig. 1B-a). We call the temporal application of these blockers under the isotonic condition ‘benzamil-pulse or amiloride-pulse’. The bathing solutions were supplemented with BFA to minimize the protein translocation from the trans-Golgi network (TGN) during this experiment. In control (without application of benzamil or amiloride), the hypotonicity caused a 5.6-fold increase in I_{SC} and this increase in I_{SC} was abolished by application of 1 μ M benzamil to the apical solution 60 min after the hypotonic stimulus (open circles in Fig. 1B-b),

Fig. 1. Usability of benzamil for labeling ENaCs in recycling. A) Dose-dependent effects of benzamil ($n = 4$) and amiloride ($n = 4$) on I_{SC} in A6 cells pretreated overnight with 1 μ M aldosterone. B) (a) Application protocol of blocker-pulse followed by hypotonic stress. ISO and HYPO refer to isotonic and hypotonic conditions, respectively. Each blocker (amiloride (10 μ M) or benzamil (1 μ M)) was applied under the isotonic condition only 120 s just before exposed to the hypotonic condition. (b) Effects of blocker-pulses on the hypotonicity-induced I_{SC} (0.1 % DMSO-pulse, open circles, $n = 4$; 10 μ M amiloride-pulse, closed squares, $n = 4$; 1 μ M benzamil-pulse, closed circles, $n = 4$). (c) Summarized data of effects of blocker-pulses on the hypotonicity-induced I_{Na} measured as benzamil-sensitive I_{SC} at 60 min after the hypotonic stress.

suggesting that the hypotonicity-induced I_{SC} is due to the ENaC-mediated I_{Na} . The hypotonicity-induced I_{Na} was abolished by benzamil-pulse (closed circles (BNZ) in Fig. 1B-b) and was partially diminished by amiloride-pulse (closed squares (AMI) in Fig. 1B-b). These observations are summarized in Fig. 1B-c. Since block of ENaC by benzamil does not disrupt trafficking [8], benzamil-labeled

ENaCs in cytosolic space would be recycled to the apical surface to the same extent as the benzamil-free ENaCs by hypotonic stress. However, we did not observe a hypotonicity-induced increase in I_{sc} in a benzamil-labeled experiment (closed circles (BNZ) in Fig. 1B-b). The observation suggests that in A6 cells benzamil is not dissociated from ENaC during these experiments due to its low rate of dissociation from ENaC, even if benzamil-labeled ENaCs are recycled to the apical surface. Consistent with this idea, amiloride with a high rate of dissociation from ENaC seems to be somewhat released from ENaC, because amiloride-pulse only partially diminished hypotonicity-induced I_{Na} (closed squares (AMI) in Fig. 1B-b). These observations indicate that: in A6 monolayers, (1) hypotonic stress stimulates ENaC trafficking from the intracellular recycling pool to the apical surface, (2) benzamil-pulse irreversibly blocks and thereby successfully labels ENaCs in recycling, and (3) under an isotonic condition, most of ENaCs are recycled between the apical surface and the intracellular channel pool within 120 s (2 min).

Estimation of the total amount of ENaCs in recycling: I_{Na} corresponds to the amount of ENaCs in the apical surface (S_{ENaC}), which depends on the total amount of ENaCs in recycling (T_{ENaC}), and the recycling rates. In the simplest form of recycling (Fig. 2), S_{ENaC} is described as follows:

$$T = S(t) + P(t) \quad (1),$$

$$\frac{dS(t)}{dt} = \alpha P(t) - \beta S(t) \quad (2),$$

where t is time, T is T_{ENaC} (the total amount of ENaCs in recycling), $S(t)$ is the amount of ENaCs in the apical membrane (S_{ENaC}) at time t , $P(t)$ is the amount of ENaCs in the intracellular channel pool (P_{ENaC}) at time t , and α and β refer respectively to the rates of insertion and endocytosis of ENaCs into and from the apical membrane. Therefore, the following eq(3) is induced from eq(1) and eq(2):

$$S(t) = \frac{\alpha}{\alpha + \beta} T + (S_0 - \frac{\alpha}{\alpha + \beta} T) \cdot e^{-(\alpha + \beta)t} \quad (3),$$

where S_0 is the value of $S(0)$. When t is infinite,

$$S(\infty) = \frac{1}{1 + \frac{\beta}{\alpha}} T \quad (4).$$

Application of hypotonic stress gradually increased I_{Na} (i.e., S_{ENaC}) with time (open circles in Fig. 1B-b). To study recycling of ENaCs, we separated I_{Na} mediated by recycled ENaCs from I_{sc} using benzamil-labeling for 0

Fig. 2. A model of ENaC recycling between the apical surface and the intracellular channel pool. α and β are respectively the rates of insertion and endocytosis of ENaCs. $S(t)$ and $P(t)$ are the amount of ENaCs located in the apical surface and in the intracellular recycling pool, respectively. T_{ENaC} is the sum of $S(t)$ and $P(t)$, representing the total amount of ENaCs in recycling.

or 30 min (Fig. 3A-a). Fig. 3A-b shows the hypotonicity-induced I_{sc} with (30 min; open circles) and without (0 s; closed circles) benzamil-pulse. We recognized the difference between hypotonicity-induced I_{sc} with and without benzamil-pulse as the I_{Na} mediated by ENaCs inserted into the apical membrane (Fig. 3A-b). The difference is plotted in Fig. 3A-c and was fitted by the eq(3), yielding $S(\infty)$ of $3.35 \mu A/cm^2$. Fig. 3B-a shows the effect of benzamil on I_{Na} under the hypotonic condition. The I_{Na} (Fig. 3B-a) was not recovered even by washing out benzamil briefly applied (20 s) (block of S_{ENaC}) when the hypotonicity-induced I_{sc} reached its peak irrespective of BFA treatment (Fig. 3B-a). This suggests that under the hypotonic condition most of ENaCs are labeled by brief application of benzamil, indicating that most of the channels are located in the apical surface but not in the intracellular channel pool under the hypotonic condition. Therefore, under the hypotonic condition $S(\infty)$ is regarded as T_{ENaC} . According to eq(4), hypotonicity is considered as a stimulus that causes a marked reduction in β/α nearly to 0. Furthermore, since S_{ENaC} ($= I_{Na}$, $0.24 \mu A/cm^2$) under

Fig. 3. Estimation of T_{ENaC} using benzamil-pulse and hypotonic stress. **A)** (a) and (d) Protocols to estimate T_{ENaC} , (b) and (e) hypotonicity-induced I_{sc} obtained by the protocols, and (c) and (f) benzamil-pulse-sensitive component of I_{Na} in the control ((a)-(c), $n = 4$) and the BFA-pretreated ((d)-(f), $n = 4$) cells. Data points in (c) and (f) were fitted by eq(2) and the fitted curves were superimposed. **B)** (a) Effects of brief application (20 s) of benzamil on the hypotonicity-induced I_{Na} in the control (closed circles, $n = 3$) and the BFA-treated cells (open circles, $n = 3$). A6 cells were incubated in the hypotonic solution with BFA after 60 min incubation in the isotonic solution without (control, closed circles) or with (BFA, open circles) BFA. In both cases (control and BFA), A6 cells were apically applied with 1 μM benzamil for 20 sec (black horizontal bar) followed by an extensive apical wash (white horizontal bar, 5-fold volume exchange). (b) Effects of BFA (5 $\mu\text{g}/\text{ml}$) on I_{Na} under isotonic ($n = 4$) and hypotonic ($n = 5$) conditions. ISO; A6 cells were incubated in the presence and absence of BFA for 60 min in the isotonic solution 180 min after incubation in the isotonic solution without BFA. The effect of BFA on I_{Na} was estimated 60 min after application of BFA compared with I_{Na} in the absence of BFA in the isotonic solution. HYPO; A6 cells were incubated in the presence and absence of BFA for 60 min in the hypotonic solution 180 min after incubation in the hypotonic solution without BFA. The effect of BFA on I_{Na} was estimated 60 min after application of BFA compared with I_{Na} in the absence of BFA in the hypotonic solution. *, $p < 0.001$. NS, no significant difference.

the isotonic condition was estimated 14% of $S(\infty)$ (3.35 $\mu\text{A}/\text{cm}^2$, Fig. 3A-c), we could consider that 14% of ENaCs are located in the apical membrane under the isotonic condition. This number is in agreement with previous results obtained in A6 cells [24], where it is calculated that $\sim 13\%$ of all ENaC α subunits is expressed in the plasma membrane.

Degradation of ENaC under the isotonic and hypotonic conditions: T_{ENaC} is dynamically balanced by

supply of newly-synthesized channels from TGN and loss of channels via degradation process. Our protocol of T_{ENaC} estimation consists of co-application of the hypotonic stimulation and BFA for 60 min (Figs. 1, 3 and 4). Thus, supply of newly-synthesized ENaCs is continuously blocked by BFA during the estimation process. On the other hand, $S(\infty)$ and thereby T_{ENaC} might be underestimated due to ENaC degradation that may occur during the T_{ENaC} estimation process. Therefore, we

Fig. 4. Estimation of the recycling rates of ENaCs. A) (a) and (d), protocols to estimate α , where the duration of benzamil-pulse is varied between the indicated range (0 – 30 min in (a) and 0 – 10 min in (d)); (b) and (e), hypotonicity-induced I_{sc} obtained by the protocols shown in (a) and (d), respectively; (c) and (f), benzamil-sensitive components of I_{sc} in the control (c) and the BFA-pretreated cells (f), respectively. Dots in (c) and (f) were fitted by eq(2) and the fitted curves were superimposed. $n = 3 - 5$. B) Decay of $S(\infty)$ against the duration of benzamil-pulse in the control (*closed circles*) and the BFA-treated (*open circles*) cells. Single-exponential fitting was made to the data and the fitted curves were superimposed.

examined the effects of BFA on I_{Na} under the hypotonic condition. Since I_{Na} (i.e., S_{ENaC}) is proportional to T_{ENaC} at constant recycling rates (steady state) (Fig. 2; $S_{ENaC} = T_{ENaC} (\alpha/(\alpha + \beta))$), we use I_{Na} for a quantitative measure of T_{ENaC} . α and β probably get settled 3 hours after the hypotonic stimulation, and most of ENaCs would be located in the apical membrane. Therefore, I_{Na} can be used as a measure of T_{ENaC} under this condition. Then, A6 cells were bilaterally applied with BFA or vehicle (0.1 % ethanol) for 60 min. BFA did not significantly affect I_{Na} under the hypotonic condition (HYPO in Fig. 3B-b), suggesting that T_{ENaC} is not significantly degraded for the duration we applied hypotonic stress for estimation of T_{ENaC} (60 min). This observation supports that we could obtain T_{ENaC} by estimating $S(\infty)$ from the observed I_{sc} for 60 min after hypotonic stimulation.

Similarly, we examined the effects of BFA on I_{Na} under the isotonic condition. BFA decreased I_{Na} to

53 % of the control value under the isotonic condition (ISO in Fig. 3B-b). This observation not only provides positive control of BFA effect but also suggests that ENaC degradation occurs under the isotonic condition. Thus, ENaC degradation more easily occurs in the isotonic condition than in the hypotonic condition. We presume this is due to the difference in the distribution of ENaCs under the hypotonic condition from the isotonic condition. As described above, most of recycled ENaCs are located in the intracellular pool under the isotonic condition, but in the apical surface under the hypotonic condition. Taken together, these observations indicate that only ENaCs in the intracellular channel pool can undergo degradation. We take advantage of this phenomenon when we use the hypotonic stress to estimate T_{ENaC} . In other words, co-application of hypotonic stress and BFA respectively abolishes both degradation and supply of the newly-synthesized channels, keeping T_{ENaC} constant during

60 min of the estimation process.

Pretreatment of brefeldin A decreases T_{ENaC} under the isotonic condition: As suggested in Fig. 3B-b, T_{ENaC} could be affected by BFA under the isotonic condition. The concept is that all the channels pass through the TGN to enter the flow of channel recycling and BFA disrupts channel trafficking from the TGN [25, 26]. Here, we examined if BFA actually decreases T_{ENaC} . A6 cells were pretreated with BFA (5 $\mu\text{g/ml}$) for 60 min under the isotonic condition before application of hypotonic stress (Fig. 3A-d). Then, T_{ENaC} was estimated using benzamil-labeling and hypotonic stress in the same way as described above (Fig. 3A-d). As a result, T_{ENaC} in the BFA-pretreated cells was estimated to be $1.36 \pm 0.17 \mu\text{A/cm}^2$ ($n = 4$, Figs. 3A-e and f). Since T_{ENaC} in control cells was estimated to be $3.35 \pm 0.38 \mu\text{A/cm}^2$ ($n = 4$, Fig. 3A-c), T_{ENaC} was significantly ($p < 0.005$) decreased to 41 % of the control by BFA treatment for 1 h under the isotonic condition, indicating that 59% of total ENaCs was degraded. The rate of degradation obtained in the present study (59 % degradation of total ENaC for 60 min) is close to the rate of degradation of metabolically radiolabeled ENaC proteins obtained in A6 cells: the half-life of the total pool of ENaC subunits were 40 – 70 min [27]. This is also in good agreement with the result in Fig. 3B-b.

Estimation of recycling rates based on the time course of benzamil-labeling: Next, we investigated how T_{ENaC} and rates of recycling interact each other. To estimate α (the rate of insertion) and β (the rate of endocytosis) shown in Fig. 2, we studied the time course of $F(t)$ decay caused by application of apical benzamil block, where $F(t)$ is an amount of ENaCs free from benzamil-binding (i.e., unlabeled ENaCs) among T_{ENaC} . The ENaCs inserted into the apical membrane are functionally blocked by apical application of benzamil. As described above, the benzamil-blocked ENaCs do not contribute to I_{SC} , even if they are recycled into the apical membrane after endocytosis. Therefore, during the time of ENaCs' recycling, apical application of benzamil decreases the amount of functional ENaCs ($F(t)$) in a manner dependent on the application period of benzamil. When we apply apical benzamil-block, F_0 (i.e., $F(t)$ at $t = 0$) is equal to the amount of ENaCs in the intracellular channel pool at $t = 0$. Based on this phenomenon, when benzamil is applied apically at $t = 0$, $F(t)$ decreases in a manner dependent on the insertion rate (α) as follows:

$$F_0 = T - S_0 \quad (5),$$

$$\frac{dF(t)}{dt} = -\alpha F(t) \quad (6),$$

where t is the duration of benzamil application, and F_0 is an amount of ENaCs free from benzamil at $t = 0$ (i.e., ENaCs in the intracellular channel pool at $t = 0$). The following eq(7) is induced from eq(5) and eq(6):

$$F(t) = (T - S_0) \cdot e^{-\alpha t} \quad (7).$$

Experimentally, $F(t)$ corresponds to $S(\infty)$ of hypotonicity-induced I_{Na} . Figs. 4A-a and d are the protocols of experiments for the control and the BFA-pretreatment, respectively. When the duration of benzamil-pulse was varied within the indicated range, $S(\infty)$ of hypotonicity-induced I_{Na} was decreased as the duration of benzamil-pulse increased (Figs. 4A-b, c, e and f). We plotted $S(\infty)$ against the duration of benzamil-pulse in Fig. 4B. Single-exponential fitting was made to the dots and fitted curves were superimposed in Fig. 4B. The time constants of the decay of $S(\infty)$ ($= F(t)$) in control and BFA-treated cells are 273 s and 73 s, respectively. According to eq(7), the reciprocal of the time constant of decay of $S(\infty)$ ($= F(t)$) is α . Therefore, α in control cells is 0.0037 s^{-1} (0.22 min^{-1}) and α in BFA-treated cells is 0.0137 s^{-1} (0.82 min^{-1}). Moreover, in the steady state under an isotonic condition the relationship between α and β is as follows:

$$\alpha \cdot P_i = \beta \cdot S_i \quad (8),$$

where S_i and P_i respectively refer to the steady-state values of $S(t)$ and $P(t)$ under an isotonic condition. Therefore, β is calculated from eq(1) and eq(8) by:

$$\beta = \frac{T - S_i}{S_i} \alpha \quad (9).$$

β in the control cells is 0.0238 s^{-1} (1.43 min^{-1}) and β in the BFA-treated cells is 0.0639 s^{-1} (3.83 min^{-1}). The recycling rate is much larger in A6 cells (insertion rate, α , is 0.22 min^{-1} and endocytosis rate, β , is 1.43 min^{-1}) compared with observations previously reported in mouse cortical collecting duct cells [8] (insertion rate is 0.12 min^{-1} and endocytosis rate is 0.15 min^{-1}), although models of recycling and types of cells used for these studies are different. According to our model, the distribution of ENaC in the apical membrane surface is determined based on the insertion and endocytosis rates as follows; $S_{\text{ENaC}}/T_{\text{ENaC}} = \alpha/(\alpha + \beta)$. The small value of $\alpha/(\alpha + \beta)$ ($= 0.14$) in A6 cells means a large amount of ENaC distributed in the intracellular pool under the isotonic condition; 14% of total ENaCs is in the apical membrane (Fig. 3A-c). On the other hand, in mouse cortical collecting duct cells, the larger value of $\alpha/(\alpha + \beta)$ ($= 0.44$) would mean an even distribution of ENaC in the apical membrane surface and the intracellular pool.

	T_{ENaC} ($\mu\text{A}/\text{cm}^2$)	S_{ENaC} ($\mu\text{A}/\text{cm}^2$)	P_{ENaC} ($\mu\text{A}/\text{cm}^2$)	insertion α (min^{-1})	endocytosis β (min^{-1})
Control	3.35 $\pm$ 0.38	0.45 $\pm$ 0.10	2.90	0.22	1.43
BFA	1.36 $\pm$ 0.17**	0.24 $\pm$ 0.04*	1.12	0.82	3.83

Table 1. Estimates of the amount of ENaCs in recycling and the rates of insertion and endocytosis in an isotonic condition. ** T_{ENaC} is significantly different between control (n = 4) and BFA (n = 4) at $p < 0.005$. * S_{ENaC} is significantly different between control (n = 4) and BFA (n = 4) at $p < 0.05$. Calculations of P_{ENaC} , α , and β were detailed in the text.

All the parameters obtained in the present study are summarized in Table 1. These results indicate that α and β respectively increased 3.7- and 2.7-fold as T_{ENaC} , suggesting that the rates of ENaC recycling would be correlated with T_{ENaC} . Further, it is interesting that α increased more largely (3.7-fold) than β (2.7-fold) as T_{ENaC} decreased, suggesting that α is more dependent on a change in T_{ENaC} than β . Such difference between α and β in the dependency on T_{ENaC} could function as negative feedback machinery responding to a change in T_{ENaC} for maintenance of S_{ENaC} . The differential regulation of degradation dependent on the channel distribution described above could also play a role in countering a change in T_{ENaC} . We suggest possible mechanisms of the regulation of the recycling rates by T_{ENaC} : 1) molecular machineries involved in insertion and endocytosis contain inherent physical properties determining the relation between the rates of recycling and T_{ENaC} , and 2) there is a sensor for S_{ENaC} (or T_{ENaC}) modulating the recycling rates via regulation of some intracellular signaling cascades related to ENaC trafficking. Although further studies are needed to confirm these mechanisms, our data elucidate that the rates of insertion and endocytosis of ENaC are correlated with T_{ENaC} .

Slow response of I_{sc} to hypotonic shock: Under the isotonic condition with BFA, the insertion and endocytosis rates (α and β) of ENaC were respectively 0.82 and 3.83 min^{-1} (Table 1), indicating that the time constant of ENaC recycling under the isotonic condition with BFA was 0.22 min. In other words, under the isotonic condition, most of ENaCs are recycled between the apical surface and the intracellular channel pool within 2 min. On the other hand, it took 30 - 40 min for I_{sc} to reach a steady state after application of hypotonic shock with BFA (open circles in Fig. 1B-b). Under the hypotonic condition with BFA, the insertion and endocytosis rates (α and β) of ENaC were respectively 0.06 and ~ 0 min^{-1} . Namely, both the insertion and endocytosis rates

of ENaC ($\alpha = 0.06$ min^{-1} and $\beta = \sim 0$ min^{-1}) were much lower under the hypotonic condition than those ($\alpha = 0.82$ min^{-1} and $\beta = 3.83$ min^{-1}) under the isotonic condition. These low insertion and endocytosis rates of ENaC under the hypotonic condition caused a slow time course for I_{sc} to reach a steady state responding to hypotonic shock (open circles in Fig. 1B-b).

Low rate of washing out amiloride under the hypotonic condition: At a steady state under the isotonic condition I_{sc} with amiloride-pulse for 20 s fully recovered to the level just before application of amiloride-pulse within 3 min after washing out amiloride. This observation is consistent with earlier studies under isotonic conditions [8, 23, 28, 29]. On the other hand, at a steady state under the hypotonic condition where all ENaCs would stay at the apical surface without endocytosis, I_{sc} with amiloride-pulse for 20 s reached to the level just before application of amiloride-pulse around 20 min after washing out amiloride. A similar observation at a steady state under a hypotonic condition is reported by Jans et al. [29]. These observations indicate that the rate of washing out amiloride under the hypotonic condition is lower than that under the isotonic condition.

Slow time course of hypotonicity-induced increase in I_{sc} after amiloride-pulse: At the end of amiloride-pulse for 120 s (2 min) shown in Fig. 1B-b (closed squares), most of amiloride-bound ENaCs were already translocated into the intracellular pool but not in the apical membrane, since amiloride was applied at a steady state under the isotonic condition where most of ENaCs were recycled within 2 min and stayed in the intracellular pool (Table 1). When the cells were exposed to the hypotonic solution, amiloride-bound ENaCs staying in the intracellular pool were translocated to the apical membrane with a slow insertion rate ($\alpha = 0.06$ min^{-1}). Then, the amiloride-bound ENaCs translocated to the apical surface were exposed to the amiloride-free hypotonic solution. After the

amiloride-bound ENaCs were exposed to the amiloride-free hypotonic solution, amiloride started to be washed out under the hypotonic condition with a low rate of washing out amiloride (full washing out time = 20 min). Based on the low insertion rate ($\alpha = 0.06 \text{ min}^{-1}$) of ENaC with the low rate of washing out amiloride (full washing out time = 20 min) under the hypotonic condition, I_{sc} after amiloride-pulse for 120 s under the isotonic condition slowly increased responding to a hypotonic shock (closed squares in Fig. 1B-b). Compared with the time course of I_{sc} increase responding to hypotonic shock without amiloride-pulse (open circles in Fig. 1B-b), the time course of I_{sc} increase responding to hypotonic shock with amiloride-pulse (closed squares in Fig. 1B-b) was slower. This slower time course would be due to the low rate of washing out amiloride (full washing out time = 20 min) under the hypotonic condition.

Conclusion

In the present study, we observed a novel regulation of ENaC recycling by T_{ENaC} : (1) the rates of insertion

(α) and endocytosis (β) increase when T_{ENaC} decreases, and (2) the difference between the sensitivities of α and β to T_{ENaC} forms a possible feedback mechanism that maintains S_{ENaC} within a proper range. Our results also suggest that over-expression of tagged-channels often used for studying channel recycling would significantly affect the natural recycling properties of the channel via an increase in the total amount of the channels in recycling. Thus, the analysis of blocker-labeled channels established in the present study leads us to further investigation on recycling of endogenously expressed ENaCs.

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