Progestosterone Down-regulates the Open Probability of the Amiloride-sensitive Epithelial Sodium Channel via a Nedd4-2-dependent Mechanism*

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Activation of the mitogen-activated protein (MAP) kinase cascade by progestrone in Xenopus oocytes leads to a marked downregulation of activity of the amiloride-sensitive epithelial sodium channel (ENaC). Here we have studied the signaling pathways involved in progestosterone effect on ENaC activity. We demonstrate that: (i) the truncation of the C termini of the αβγENaC subunits results in the loss of the progestosterone effect on ENaC; (ii) the effect of progestosterone was also suppressed by mutating conserved tyrosine residues in the Pro-X-X-Tyr (PY) motif of the C termini of the β and γ ENaC subunits (P613A and Y622A); (iii) the down-regulation of ENaC activity by progestosterone was also suppressed by co-expression ENaC subunits with a catalytically inactive mutant of Nedd4-2, a ubiquitin ligase that has been previously demonstrated to decrease ENaC cell-surface expression via a ubiquitin-dependent internalization/degradation mechanism; (iv) the effect of progestosterone was significantly reduced by suppression of consensus sites (P613A and Y623A) for ENaC phosphorylation by the extracellular-regulated kinase (ERK), a MAP kinase previously shown to facilitate the binding of Nedd4-2 ubiquitin ligases to ENaC; (v) the quantification of cell-surface-expressed ENaC subunits revealed that progestosterone decreases ENaC open probability (whole cell P0, wcP0) and not its cell-surface expression. Collectively, these results demonstrate that the binding of active Nedd4-2 to ENaC is a crucial step in the mechanism of ENaC inhibition by progestosterone. Upon activation of ERK, the effect of Nedd4-2 on ENaC open probability can become more important than its effect on ENaC cell-surface expression.

The amiloride-sensitive epithelial sodium channel (ENaC) is a highly selective Na⁺ channel found in the apical membrane of salt-reabsorbing tight epithelia, including the kidney distal nephron, the distal colon, the salivary and sweat glands, and the lung. ENaC activity is essential for maintaining extracellular fluid volume and blood pressure. The activity of this channel is controlled at two levels: the number of active channels on the cell surface (N) and the channel open probability (P0) (1). However, quantitative estimates of N and P0 values remain difficult, mainly because of the low cell-surface expression and high variability of the open probability of ENaC. Aldosterone, the principal hormone controlling ENaC activity, has been shown to have a major effect on N. One of the molecular mechanisms proposed for the regulation of ENaC cell-surface expression by aldosterone involves Nedd4-2 ubiquitin ligase and serum- and glucocorticoids-induced kinase isoform 1 (sgk1). In a model proposed by Debonneville et al. (2) for the principal cell of the kidney collecting duct, binding of Nedd4-2 to the Pro-X-X-Tyr (PY) motif in the β and γ ENaC subunits results in ENaC ubiquitylation and intracellular degradation. Stimulation of sgk1 expression by aldosterone provokes sgk1-dependent Nedd4-2 phosphorylation, resulting in a decreased binding affinity of Nedd4-2 to ENaC and, finally, in an increased ENaC number at the cell surface. However, recent evidence indicates that increased sgk1 expression may also be involved in the regulation of ENaC P0. Diakov et al. (3) have shown that sgk1 can directly phosphorylate the C terminus of the αENaC subunit, and that this phosphorylation significantly increases ENaC activity via an increase in channel P0. Alvarez de la Rosa et al. (4) have recently demonstrated that expression of a constitutively active mutant of sgk1 in A6 cells results in a significant increase of both N and P0. Using measurements of wcP0, a similar dual effect of sgk1 on ENaC N and P0 was observed by Vuagniaux et al. (5) in Xenopus oocyte expression system. We have also shown that a naturally occurring mutation of the PY motif (P613A and Y623A), which causes an autosomal dominant form of salt-sensitive hypertension (Liddle syndrome), results in both an increase in N and predominant change in P0 (6). Collectively, these data indicate that sgk1- and/or Nedd4-2-dependent mechanisms may control ENaC activity by controlling both the N and P0.

Another mechanism known to play an important role in the control of ENaC activity is the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) signaling pathway. This signaling pathway is thought to have a dual effect on ENaC. The long term (hours/days) activation of this pathway leads to the down-regulation of ENaC activity via inhibition of the transcription rate of ENaC subunits (7, 8). The short term (minutes) activation of ERK1/2 also down-regulates ENaC activity. The molecular mechanism responsible for this acute effect remains poorly understood. Shi et al. (9) have shown that ERK is capable of directly phosphorylating the β and γ ENaC subunits on residues Thr-613 and Thr-623, respectively. The phosphorylation of β613 γ623 increases the affinity of WW domains of Nedd4-1 ubiquitin ligase (a closely related homologue of Nedd4-2) for the C termini of the β and γ subunits. β613 and γ623 mutations significantly increased the β613 γ623 affinity but whether changes in N and/or P0 were responsible for this increase was not determined (9). We have recently shown that activation of ERK pathway by progesterone in Xenopus oocytes leads to a marked downregulation of ENaC activity (10). However, our data did not fit well with a Nedd4-2-dependent ENaC ubiquitylation/degradation mechanism, because quantification of ENaC expression at the cell surface revealed that the main effect of progesterone is a decrease of channel P0. The aim of this study was to investigate whether a Nedd4-2-dependent mechanism could be involved in the progesterone effect.
of the present study was to analyze the molecular mechanisms of the observed \( P_a \) effect. Detailed analysis of the progesterone effect on ENaC activity demonstrated, contrary to our expectations, that the regulation of ENaC \( P_a \) by progesterone is PY motif- and Nedd4-2-dependent. These results demonstrate a novel mode of action of Nedd4-2 in the regulation of channel function by \( P_a \).

**EXPERIMENTAL PROCEDURES**

Expression of ENaC and Nedd4-2 in Xenopus oocytes—Complementary RNAs for rat \( \alpha \beta \gamma \) ENaC subunits, human Nedd4-2, and mouse NaPi-IIa sodium phosphate co-transporter were synthesized in vitro using SP6 polymerase. The cRNAs were injected into Xenopus oocytes (10 ng of total cRNA/oocyte), and injected oocytes were kept in modified Barth solution (MBS) containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO\(_3\), 0.8 mM MgSO\(_4\), 0.3 mM Ca(NO\(_3\))\(_2\), 0.4 mM CaCl\(_2\), 10 mM Hepes-NaOH (pH 7.2). Electrophysiological measurements and cell surface biotinylation were performed 24 h after injection. Macromscopic amiloride-sensitive Na\(^+\) current (\( I_{Na}\)) was defined as the difference between Na\(^+\) current obtained in the presence (5 \( \mu \)M) and in the absence of amiloride and was recorded, using the two-electrode voltage clamp method. The mean \( I_{Na}\) values in different oocyte batches ranged between 1 and 15 \( \mu \)A.

Biotinylation of ENaC Subunits on the Cell Surface—Biotinylation was performed in 48-well plates, using 20 oocytes per experimental condition. All biotinylation steps were performed in a cold room, using ice-cold solutions. After incubation in ice-cold MBS solution for 30 min, the oocytes were washed three times with MBS. After the last wash, the MBS solution was removed and replaced by a biotinylation buffer containing 10 mM triethanolamine, 150 mM NaCl, 2 mM CaCl\(_2\), 1 mg/ml ice-cold solutions. After incubation in ice-cold MBS solution for 30 min, was performed in 48-well plates, using 20 oocytes per experimental condition, and injected oocytes were kept in modified Barth solution (MBS) containing: 1% Triton X-100, 500 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl (pH 7.5) added to the MBS solution. After the overnight incubation at 4 °C with shaking, the tubes were centrifuged for 10 min at 12,000 rpm. Supernatants were transferred to the new 1.5-ml Eppendorf tubes containing 50 \( \mu \)l of immunopure immobilized streptavidin beads (Pierce) washed with lysis buffer. After overnight incubation at 4 °C with shaking, the tubes were centrifuged for 1 min at 5000 rpm. Supernatant was removed, and beads were washed three times with lysis buffer. 60 \( \mu \)l of SDS-PAGE sample buffer was added to the beads. All samples were heated for 5 min at 95 °C before loading on the 8% SDS-PAGE.

**RESULTS**

The Effect of Progesterone on ENaC Activity Is PY Motif- and Nedd4-2-dependent—As shown in Fig. 1, the addition of progesterone (15 \( \mu \)M) to ENaC-expressing oocytes leads to a significant down-regulation of amiloride-sensitive Na\(^+\) current (\( I_{Na}\)). The \( I_{Na}\) is inhibited rapidly; ~50% of \( I_{Na}\) inhibition is reached within 30 min of progesterone treatment. The short-time required for \( I_{Na}\) inhibition indicated that ENaC activity and/or cell-surface expression, rather than cRNA stability or protein synthesis, are regulated by progesterone. This was confirmed by the stable expression of \( \alpha \beta \gamma \)ENaC subunit proteins, over 4 h of treatment with progesterone (see below). To assess which parts of the ENaC subunits are involved in channel responsiveness to progesterone, we first expressed \( \alpha \beta \gamma \)ENaC subunits with truncated C termini. For the \( \alpha \) truncation we used a mutant in which all C-terminal amino acids were eliminated (\( \alpha_{\beta618\alpha\tau\beta} \)). For the \( \beta \) and \( \gamma \) subunits truncation, we used the \( \beta_{\beta665\alpha\tau\beta} \) and \( \gamma_{\gamma574\alpha\tau\beta} \) mutants that cause Liddle disease (11). As shown in Fig. 2A, the truncation of \( \alpha \beta \gamma \) C termini results in a complete loss of the progesterone effect on ENaC. The \( \alpha \) truncation in the C terminus of \( \alpha \) and \( \gamma \) subunits have been demonstrated as the important regulatory elements of ENaC activity, we next assessed the effect of progesterone on the \( \beta_{\beta618\alpha\tau\beta} \) and \( \gamma_{\gamma628\alpha\tau\beta} \) mutants. As shown in Fig. 2B, the mutation of the \( \beta \) and \( \gamma \) subunits truncation, either alone or together, resulted in the complete suppression of the progesterone effect on ENaC. This result clearly demonstrated that the progesterone effect on ENaC is dependent on PY motif of either \( \beta \) or \( \gamma \) (but not \( \alpha \)) ENaC subunits. The \( \gamma \) PY motifs are required for clathrin-mediated ENaC internalization.
mutant (Nedd4-2CS mutant) on channel activity. As shown in Fig. 2C, co-expression of ENaC with the Nedd4-2CS mutant leads to a complete loss of the progesterone effect. As expected, PY mutations (Fig. 2B) or co-expression with the Nedd4-2CS mutant (Fig. 2C) both resulted in a significant increase in I Na. Collectively, these experiments demonstrated that elimination of Nedd4-2 binding domains in ENaC subunits or suppression of endogenous ubiquitin ligase activity by expression of the Nedd4-2CS mutant lead to the abolishment of the progesterone effect on ENaC.

The Effect of Progesterone on ENaC Activity Is Reduced by Mutation of the Consensus Sites for ERK Phosphorylation—Recently, several amino acids lying outside the PY motifs have been proposed to be involved in the interaction between ENaC and WW-domains of Nedd4–1 ubiquitin ligase, a closely related homolog of Nedd4-2. Because the effect of progesterone on ENaC depends on the activation of the ERK pathway (10), we also tested the effect of these two phospho-

![Figure 2](image)

**Figure 2.** Progesterone down-regulates ENaC activity via PY motifs and Nedd4-2-dependent mechanism. A, expression of αβγENaC subunits with truncated C termini (αβγENaC(Pro649Tyr762/Trp762)) resulted in the loss of the progesterone effect on I Na. Oocytes were treated with 15 μM for 4 h. Shown are mean ± S.E. of eight independent experiments each performed with five oocytes per experimental condition. *, statistical significance of <0.05. B, expression of αβγENaC subunits with mutated PY motifs in β (β(Y618A) and γ (γ(Y628A)) subunits also results in the loss of the progesterone effect on I Na. Oocytes were treated with 15 μM for 4 h. Shown are mean ± S.E. of seven independent experiments each performed with five oocytes per experimental condition. *, statistical significance of <0.05. C, co-expression of ENaC with a catalytically inactive mutant of Nedd4-2 (Nedd4-2CS) results in abolishment of the progesterone effect on I Na. Oocytes were treated with 15 μM for 4 h. Shown are mean ± S.E. of eleven independent experiments each performed with five oocytes per experimental condition. *, statistical significance of <0.05. **, statistical significance of <0.001.

![Figure 3](image)

**Figure 3.** β-Tyr-613 and γ-Tyr-623 residues are involved in the progesterone effect on ENaC. A, expression of β-Tyr-613 (β(Y618A)) or γ-Tyr-623 (γ(Y628A)) mutants results in a significant reduction of the inhibitory effect of progesterone on I Na. Shown are mean ± S.E. of eleven independent experiments each performed with five oocytes per experimental condition. **, statistical significance of <0.001. B, progesterone increases phosphorylation of the βENaC subunit. Oocytes were co-injected with FLAG-tagged αβγENaC cRNAs and NaPiIIa cRNA. 24 h after injection, oocytes were loaded for 4 h with [32P]orthophosphate. Immunoprecipitation was performed in non-denaturing conditions, using anti-FLAG antibody. C, progesterone-induced phosphorylation of βENaC is abolished in βγγγ γ mutant. Oocytes were co-injected with NaPiIIa cRNA and αβγENaC or αβγP(Y613A)ENaC FLAG-tagged cRNAs. 24 h after injection, oocytes were loaded for 4 h with [32P]orthophosphate. Immunoprecipitation was performed in non-denaturing conditions, using anti-FLAG antibody.
with NaPiIIa allowed the detection of βENaC phosphorylation after co-immunoprecipitation of all three αβγ subunits in non-denaturing conditions. Statistical analysis of six independent experiments revealed a 30 ± 8% (p = 0.05) increase in βENaC phosphorylation under progesterone treatment. Mutation of Thr-613 to alanine significantly reduced the basal βENaC phosphorylation and abolished progesterone-dependent βENaC phosphorylation (Fig. 3C). The phosphorylation of α and γ ENaC subunits was not detectable, potentially due to the complex pattern of α and γ ENaC processing by endogenous proteases (see below). Collectively, these results demonstrate that elimination of extra-PY motif binding sites for Nedd4 ubiquitin ligases reduces the effect of progesterone on ENaC.

Development of a New Assay for Quantification of Channel N—It has been proposed that the main effect of Nedd4-2 on ENaC activity consists of the reduction of channel N. However, our previous experiments demonstrated that the main effect of progesterone is a decrease of channel wcP4 (10). The effect of progesterone on wcP4 was previously determined, using an assay based on the binding of an iodinated monoclonal antibody to a FLAG epitope introduced in the extracellular loops of αβγENaC subunits (6). To avoid some difficulty inherent to a binding assay, we have developed an independent procedure to quantitate N. This procedure is based on the biotinylation of cell-surface-expressed ENaC subunits (see “Experimental Procedures”). The biotinylated proteins were precipitated with streptavidin-agarose beads and electrophoresed through SDS-PAGE. In parallel, 10% of intracellular proteins recovered after precipitation of biotinylated proteins were loaded on the same gel. The proteins were blotted with anti-α, anti-β, and anti-γ ENaC antibodies. As a control for the biotinylation procedure, the same blots were re-probed with an anti-actin antibody. To validate this procedure, we quantified the cell-surface expression of the wild-type channel and the channel composed of the wild-type α and β subunits and the γ subunit with mutated PY motif (Y628A mutant). The use of the γ subunit rather than the β subunit mutant was chosen, because the epitope for the anti-β subunit antibody encompassed the PY motif. As shown in Fig. 4A, the anti-α antibody specifically recognizes several molecular species of the α subunit expressed at the cell surface: a band of ~82 kDa corresponding to the glycosylated form of full-length αENaC protein, a band of ~65 kDa and the double bands of ~25 kDa. Because the epitope for this antibody is located in the N-terminal part of αENaC, the double bands of ~25 kDa could correspond to the N-terminal products of furin-dependent αENaC cleavage described by Hughey et al. (15). The ~65-kDa band represents an as-yet uncharacterized αENaC protein species, but could correspond to a species of ~65 kDa observed in airway cells, using a similar protocol and the same antibody (16, 17). The intensity of both the ~82-kDa band and the ~25-kDa double bands was significantly increased in αβγY628A channel. In the intracellular protein pool, the αENaC antibody recognizes two αENaC species; one, which is identical to the ~65-kDa band of the cell-surface-expressed α subunit and the second one, which is migrating at ~78 kDa. A possible explanation for the difference between αENaC migration profile in the biotinylated and non-biotinylated protein pools consists of the presence in the intracellular pool of important amounts of yolk proteins migrating in a range of molecular masses of ~80–100 kDa (see Ponceau red protein staining in Fig. 4D). This large amount of yolk proteins in oocytes could be a confounding factor for the migration of other intracellular proteins in this range of molecular masses, including the 82-kDa band of αENaC.

As shown in Fig. 4B, the anti-β antibody specifically recognizes two molecular species of βENaC expressed at the cell surface: a major band of ~110 kDa that corresponds to the glycosylated form of full-length βENaC protein and an as-yet uncharacterized minor band of ~75 kDa. The intensity of both ~95-kDa and 75-kDa bands was significantly increased in αβγY628A channel. In the intracellular protein pool, the βENaC antibody recognizes two bands: one, which is identical to the minor ~75-kDa band, and the second one, migrating at ~100 kDa. As discussed above, the difference in migration profiles of cell surface and intracellular βENaC potentially comes from the interference between βENaC and yolk proteins migration. As shown in Fig. 4C, probing of biotinylated proteins with an anti-γENaC antibody reveals a ~85-kDa band corresponding to the glycosylated form of full-length γENaC protein and the double bands of ~63 kDa. These double bands could correspond to the furin-cleaved γENaC described by Hughey et al. (15). Cell surface expression of both the ~65-kDa band and the ~63-kDa double bands was significantly increased in αβγY628A mutant channel. Probing of intracellular proteins with the anti-γ antibody revealed that this antibody strongly cross-reacts with oocyte yolk proteins. Importantly, only intracellular protein pools were positive for anti-actin staining, thus demonstrating that only cell-surface-expressed proteins were biotinylated in our experiments (Fig. 4E). The absence of the 25-kDa

FIGURE 4. Cell surface biotinylation of ENaC subunits. A, cell-surface biotinylated proteins (left panel) and an intracellular protein pool (right panel) were probed with an anti-αENaC antibody. This antibody recognizes an N-terminal epitope in the αENaC subunit. B, cell-surface biotinylated proteins (left panel) and an intracellular protein pool (right panel) were probed with an anti-βENaC antibody. This antibody recognizes C-terminal epitope in the βENaC subunit. C, cell-surface biotinylated proteins (left panel) as well as intracellular protein pool (right panel) were probed with an anti-γENaC antibody. This antibody recognizes a C-terminal epitope in γENaC subunit. D, Ponceau red staining of Western blot membrane reveals a major band of oocyte yolk proteins migrating in 80- to 100-kDa range of molecular masses. This band potentially interferes with the migration of αβγ subunits in the intracellular protein pool. E, probing of the same Western blot with an anti-actin antibody demonstrates that cell-surface biotinylated proteins are not contaminated by the intracellular proteins.
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αENaC species and a low amount of the 63-kDa γENaC species in the intracellular pool of proteins (Fig. 4, A and C, respectively) indicate that only a minor fraction of cell-surface-expressed α and γ ENaC subunits remains non-biotinylated.

Collectively, these experiments demonstrated that the anti-α, anti-β, and anti-γ ENaC antibodies can be used for Western blot detection of cell-surface-expressed ENaC subunits. These experiments also indicate that quantitative analysis of Western blots can be used for the quantification of ENaC expression at the cell surface. However, a complex pattern of molecular species for α and γ subunits could potentially reduce the precision of estimates for these subunits. Thus, for quantitative analysis of ENaC at the cell surface, we used the β subunit, which is represented by a single major band of ∼95 kDa. As shown in Fig. 5, the αβγγ2A channel exhibits a 13-fold increase in INaNa, as compared with the wild-type channel, whereas cell-surface expression is increased only by 2.18-fold. These results fit well with the previously observed double effect of the Liddle mutation on N and P (6) and, thus, validate the use of biotinylation for quantification of ENaC expression at the cell surface.

Progesterone Down-regulates ENaC Activity by Decreasing Channel P. To assess the mechanism of the progesterone effect on ENaC activity, we performed a parallel analysis of INaNa and cell-surface expression of ENaC in oocytes treated with progesterone. As shown in Fig. 6A, 4-h treatment with progesterone resulted in an 80% inhibition of INaNa. Biotinylation experiments presented in Fig. 6B revealed no significant difference for βENaC expression, neither at the cell surface, nor in the intracellular pool. Paired statistical analysis of nine independent experiments revealed a 20% non-significant reduction in N in oocytes treated with progesterone (Fig. 6C). Interestingly, we have previously observed a 20% non-significant reduction of N under progesterone treatment by the antibody binding assay (10). Collectively, these results demonstrate that the major effect of progesterone is the decrease of channel INa

**DISCUSSION**

Nedd4-2 Has Two Distinct Effects on ENaC Function in the Xenopus Oocyte Expression System—Ubiquitylation is a common post-translational modification, consisting in the covalent transfer of one or multiple copies of ubiquitin, a 76-amino acid protein, to the target proteins. Although ubiquitylation is usually considered as a signal for protein degradation by the proteasome, the attachment of ubiquitin can also regulate protein localization and/or activity, independent of proteolysis. For example, ubiquitylation has been shown to act as a regulated localization signal for nuclear or integral membrane proteins (reviewed in Ref. 18). Ubiquitylation has also been demonstrated to control the activity of components of the endocytic machinery, the activity of transcription factors and the kinase activity (reviewed in Ref. 19).

Ubiquitylation is a multiple step reaction in which attachment of ubiquitin to the target proteins is catalyzed by ubiquitin protein ligases, or E3 enzymes. Numerous studies have demonstrated that Nedd4-2, an E3 enzyme, is capable of ubiquitylation of ENaC subunits (20, 21). This ubiquitylation has been proposed to down-regulate the activity of ENaC by decreasing the number of active channels (N) at the cell surface (22–24). This hypothesis implies that binding of Nedd4-2 to C-terminal PY motifs leads to ubiquitylation and degradation of ENaC subunits through the ubiquitin–26 S proteasome pathway. This hypothesis, however, does not take into account the fact that the suppression of ENaC-Nedd4-2 interaction in a Liddle syndrome mutant also results in a parallel increase in channel INa Na (6). To explain this double effect of PY mutation, one might propose either the existence of a Nedd4-2-inde-
Hormonal Regulation of ENaC: N versus P_o?—The N versus P_o regulation of ENaC activity in different physiological or pathophysiological situations remains a matter of controversy (reviewed in Ref. 1). We will briefly discuss the mechanisms of ENaC regulation by aldosterone in the context of the Liddle syndrome, because it involves a lack of interaction between the ENaC β and γ subunits with Nedd4-2.

Aldosterone-dependent Signaling Cascade—Dahlmann et al. (27) have observed that the mineralocorticoid regulation of ENaC was fully maintained in a mouse model of Liddle syndrome. The I Na through epithelial sodium channels was measured in the principal cell of the cortical collecting duct (CCD) of mice expressing mutated βENaC subunit. The Liddle mice had a much larger I Na, can contribute to the pathogenesis of an autosomal recessive form of a polycystic kidney disease. The mechanism of epidermal growth factor action on N and/or P_o was not reported in these studies. Our present data indicate that P_o inhibition could be the principal mechanism.

REFERENCES

1. Rossier, B. C. (2002) J. Gen. Physiol. 120, 67–70
2. Debonneville, C., Flores, S. Y., Kamynina, E., Plant, P. J., Tauxe, C., Thomas, M. A., Munster, C., Charbi, A., Pratt, J. H., Horisberger, J. D., Pearce, D., Lofing, J., and Staub, O. (2001) EMBO J. 20, 7052–7059
3. Diakov, A., and Korbmacher, C. (2004) J. Biol. Chem. 279, 38134–38142
4. Alvarez de la Rosa, D., Paunescu, T. G., Els, W. J., Helman, S. I., and Canessa, C. M. (2004) J. Gen. Physiol. 124, 395–407
5. Vaugnigna, G., Vallet, V., Jaeger, N. F., Hummeler, E., and Rossier, B. C. (2002) J. Gen. Physiol. 120, 191–201
6. Firson, D., Schild, L., Gautsche, I., Merillat, A. M., Schneeberger, E., and Rossier, B. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15370–15375
7. Shen, J. P., and Cotton, C. U. (2003) Am. J. Physiol. 284, F57–F64
8. Wang, H. C., Zentner, M. D., Deng, H. T., Kim, K. J., Wu, R., Yang, P. C., and Ann, D. K. (2000) J. Biol. Chem. 275, 8060–8069
9. Shi, H., Asher, C., Chigusa, A., Yung, Y., Reuveny, E., Seger, R., and Garty, H. (2002) J. Biol. Chem. 277, 13539–13547
10. Nicod, M., Miehlig, S., Flahaut, M., Salinas, M., Fowler-Jaeger, N., Horisberger, J. D., Rossier, B. C., and Firsov, D. (2002) EMBO J. 21, 5109–5117
11. Schild, L., Canessa, C. M., Shimkets, R. A., Gautsche, I., Lifton, R. P., and Rossier, B. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5699–5703
12. Ridley, J. L., Sodol, M., and Winder, S. J. (2002) Cell Signal. 14, 183–189
13. Shimkets, R. A., Lifton, R. P., and Canessa, C. M. (1997) J. Biol. Chem. 272, 25537–25541
14. Kanelis, V., Rotin, D., and Forman-Kay, J. D. (2001) Nat. Struct. Biol. 8, 407–412
15. Hughey, R. P., Burns, J. B., Kinlough, C. L., and Kleyman, T. R. (2004) J. Biol. Chem. 279, 48491–48494
16. Planes, C., Blot-Chabaud, M., Matthay, M. A., Couetue, S., Uchida, T., and Clerici, C. (2002) J. Biol. Chem. 277, 47318–47324

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17. Planes, C., Leyvraz, C., Uchida, T., Angelova, M. A., Vuagniaux, G., Hummler, E., Matthay, M. A., Clerici, C., and Rossier, B. C. (2005) *Am. J. Physiol.* **288**, L1099–L1109.
18. Hicke, L., and Dunn, R. (2003) *Annu. Rev. Cell Dev. Biol.* **19**, 141–172.
19. DiAntonio, A., and Hicke, L. (2004) *Annu. Rev. Neurosci.* **27**, 223–246.
20. Staub, O., Dho, S., Henry, P., Correa, J., Ishikawa, T., McGlade, J., and Rotin, D. (1996) *EMBO J.* **15**, 2371–2380.
21. Kamynina, E., Debonneville, C., Bens, M., Vandewalle, A., and Staub, O. (2001) *FASEB J.* **15**, 204–214.
22. Flores, S. Y., Debonneville, C., Staub, O., Verrey, F., Loffing, J., Zecevic, M., Heitzmann, D., and Kamynina, E. (2003) *Pflugers Arch.* **446**, 334–338.
23. Verrey, F., Loffing, J., Zecevic, M., Heitzmann, D., Staub, O., and Kamynina, E. (2003) *Cell. Physiol. Biochem.* **13**, 21–28.
24. Kamynina, E., and Staub, O. (2002) *Am. J. Physiol.* **283**, F377–F387.
25. Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997) *ENBO J.* **16**, 6325–6336.
26. Rajan, S., Plant, L. D., Rabin, M. L., Butler, M. H., and Goldstein, S. A. (2005) *Cell* **121**, 37–47.
27. Dahlmann, A., Pradervand, S., Hummler, E., Rossier, B. C., Frindt, G., and Palmer, L. G. (2003) *Am. J. Physiol.* **285**, F310–F318.
28. Loffing, J., Zecevic, M., Feraille, E., Kaisling, B., Asher, C., Rossier, B. C., Firestone, G. L., Pearce, D., and Verrey, F. (2001) *Am. J. Physiol.* **280**, F675–F682.
29. Hummler, E., Barker, P., Gatzy, J., Beermann, F., Verduzco, C., Schmidt, A., Boucher, R., and Rossier, B. C. (1996) *Nat. Genet.* **12**, 325–328.
30. Pradervand, S., Vandewalle, A., Bens, M., Gautschi, I., Loffing, J., Hummler, E., Schild, L., and Rossier, B. C. (2003) *J. Am. Soc. Nephrol.* **14**, 2219–2228.
31. Veizis, I. E., and Cotton, C. U. (2005) *Am. J. Physiol.* **288**, F474–F482.