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Progesterone 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 progesterone in *Xenopus* oocytes leads to a marked down-regulation of activity of the amiloride-sensitive epithelial sodium channel (ENaC). Here we have studied the signaling pathways involved in progesterone effect on ENaC activity. We demonstrate that: (i) the truncation of the C termini of the $\alpha\beta\gamma$ENaC subunits results in the loss of the progesterone effect on ENaC; (ii) the effect of progesterone 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 (β_{Y618A} and γ_{Y628A}); (iii) the down-regulation of ENaC activity by progesterone 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 progesterone was significantly reduced by suppression of consensus sites (β_{T613A} and γ_{T623A}) for ENaC phosphorylation by the extracellular-regulated kinase (ERK), a MAP kinase previously shown to facilitate the binding of Nedd4 ubiquitin ligases to ENaC; (v) the quantification of cell-surface-expressed ENaC subunits revealed that progesterone decreases ENaC open probability (whole cell P_o, wcP_o) 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 progesterone. 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)[2] 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 (P_o) (1). However, quantitative estimates of *N* and P_o 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 P_o. 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 P_o. 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 P_o. Using measurements of wcP_o, a similar dual effect of sgk1 on ENaC *N* and P_o 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 ($\beta_{R564stop}$), which causes an autosomal dominant form of salt-sensitive hypertension (Liddle syndrome), results in both an increase in *N* and predominant change in P_o (6). Collectively, these data indicate that sgk1- and/or Nedd4-2-dependent mechanisms may control ENaC activity by controlling both the *N* and P_o.

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 β_{T613} and γ_{T623} 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. β_{T613A} and γ_{T623A} mutations significantly increased the I_{Na}, but whether changes in *N* and/or P_o 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 down-regulation 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 P_o. The aim

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[2] The abbreviations used are: ENaC, amiloride-sensitive epithelial sodium channel; sgk1, serum- and glucocorticoids-induced kinase isoform 1; PY, Pro-*X*-*X*-Tyr motif; ERK, extracellular signal-regulated kinase; CCD, cortical collecting duct; MBS, modified Barth solution; cRNA, complementary RNA; wcP_o, whole cell P_o; E3, ubiquitin-protein isopeptide ligase; MAP, mitogen-activated protein.

of the present study was to analyze the molecular mechanisms of the observed P_o effect. Detailed analysis of the progesterone effect on ENaC activity demonstrated, contrary to our expectations, that the regulation of ENaC P_o 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_o.

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. Macroscopic amiloride-sensitive Na^+ current (I_{Na}) was defined as the difference between Na^+ current obtained in the presence (5 μM) 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 μA.

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 EZ-link sulfo-NHS-SS-Biotin (Pierce), pH 9.5. The incubation lasted 15 min with gentle agitation. The biotinylation reaction was stopped by washing the oocytes two times with a Quench buffer containing 192 mM glycine, 25 mM Tris-Cl (pH 7.5) added to the MBS solution. After the second wash, oocytes were incubated for 5 min in the Quench buffer with gentle agitation. Oocytes were then washed two times with the MBS solution and transferred to 1.5-ml Eppendorf tubes. Oocytes were lysed by repeated pipetting with Pasteur pipettes in a lysis buffer containing: 1% Triton X-100, 500 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl (20 μl/oocyte). The lysates were vortexed for 30 s, and the mixture was centrifuged for 10 min at 12,000 rpm. Supernatants were transferred to the new 1.5-ml Eppendorf tubes containing 50 μl 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 μl 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.

Antibodies—The anti-$\alpha\beta\gamma$ENaC antibodies were previously described. The anti-actin antibody was from Sigma.

ENaC Phosphorylation Assay—For maximal loading of oocytes with [^{32}P]orthophosphate (H_3PO_4), the oocytes were co-injected with FLAG-tagged $\alpha\beta\gamma$ENaC and NaPiIIa sodium phosphate co-transporter cRNAs. 24 h after injection, 20 oocytes/experimental condition were transferred to the 2-ml Eppendorf tubes. MBS was removed and replaced by 100 μl/tube MBS solution complemented with 250 μCi of [^{32}P]orthophosphate (Hartmann). Oocytes were incubated for 3 h at 19 °C, washed three times with MBS, and lysed in the lysis buffer containing: 80 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 100 mM NaF, 10 mM disodium pyrophosphate, 20 μM sodium orthovanadate. The lysates were centrifuged for 10 min at 2,500 rpm. Supernatants were transferred to the 1.5-ml Eppendorf tubes and centrifuged for 20 min at 10,000 rpm. Supernatants were discarded, and pellets were solubilized in a buffer containing: 0.5% digitonin, 100 mM NaCl, 20 mM Tris-HCl, 2 mM phenylmethylsulfonyl fluoride, 100 mM NaF, 10 mM disodium pyrophosphate, 20 μM sodium orthovanadate. Immunoprecipitation was performed in non-denaturing conditions with monoclonal anti-FLAG antibody (Sigma), as described (6). Samples were run on 8–13% SDS-PAGE, and phosphorylation of $\alpha\beta\gamma$ENaC subunits was revealed by autoradiography.

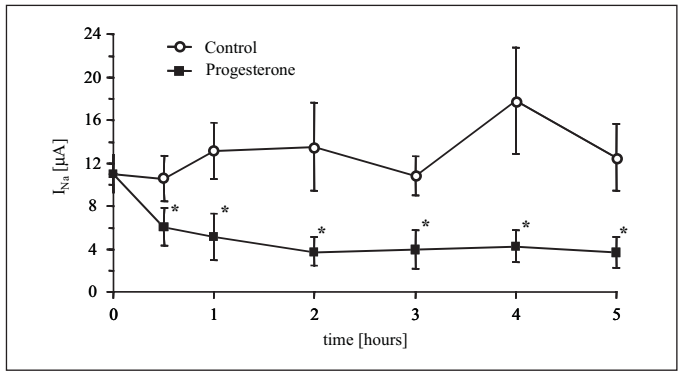

FIGURE 1. **Progesterone rapidly down-regulates ENaC activity.** Oocytes were injected with $\alpha\beta\gamma$ENaC cRNAs. The I_{Na} was measured by the voltage-clamp technique, 24 h after cRNA injection. Progesterone was dissolved in EtOH and tested at 15 μM concentration (0.15% EtOH). The equivalent amount of EtOH was added to the control oocytes. Shown are mean ± S.E. of three independent experiments each performed with five oocytes per experimental condition. *, statistical significance of <0.05.

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 μM) 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 α truncation we used a mutant in which all C-terminal amino acids were eliminated ($\alpha_{R618stop} = \alpha_T$). For the β and γ subunits truncation, we used the $\beta_{R564stop}$ (β_T) and $\gamma_{W574stop}$ (γ_T) mutants that cause Liddle disease (11). As shown in Fig. 2*A*, the truncation of $\alpha\beta\gamma$ C termini results in a complete loss of the progesterone effect on ENaC. Because the PY motifs in the C termini of β and γ subunits have been demonstrated as the important regulatory elements of ENaC activity, we next assessed the effect of progesterone on the β_{Y618A} and γ_{Y628A} mutants. As shown in Fig. 2*B*, the mutation of the PY motifs in both β and γ subunits, 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 β or γ (but not α) ENaC subunits. The PY motifs have been proposed to bind various WW-domain-containing proteins, including dystrophin, caveolins, ubiquitin ligases, and other proteins (12). Also, Shimkets *et al.* (13) have proposed that β and γ PY motifs are required for clathrin-dependent ENaC endocytosis. Because ENaC interaction with Nedd4-2 has been shown to be an important mechanism controlling ENaC activity, we next tested the effect of the catalytically inactive Nedd4-2_{C145S}

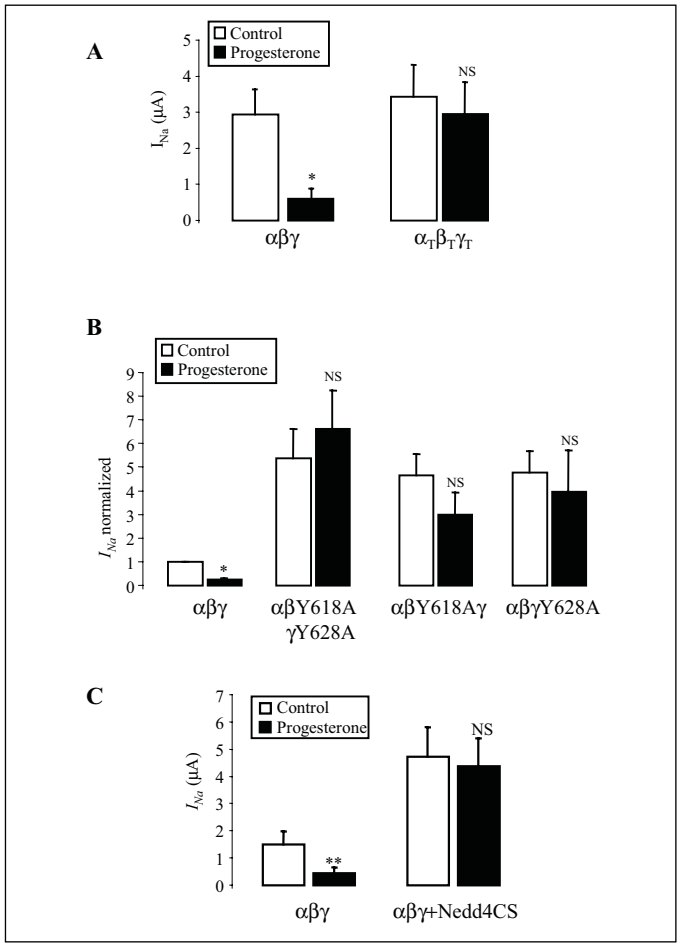

FIGURE 2. **Progesterone down-regulates ENaC activity via PY motifs and Nedd4-2-dependent mechanism.** *A*, expression of $\alpha\beta\gamma$ENaC subunits with truncated C termini ($\alpha_{R618stop}\beta_{R564stop}\gamma_{R574stop}$) resulted in the loss of the progesterone effect on I_{Na}. Oocytes 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 $\alpha\beta\gamma$ENaC 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 eleven 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 fifteen independent experiments each performed with five oocytes per experimental condition. *, statistical significance <0.05. **, statistical significance of <0.001.

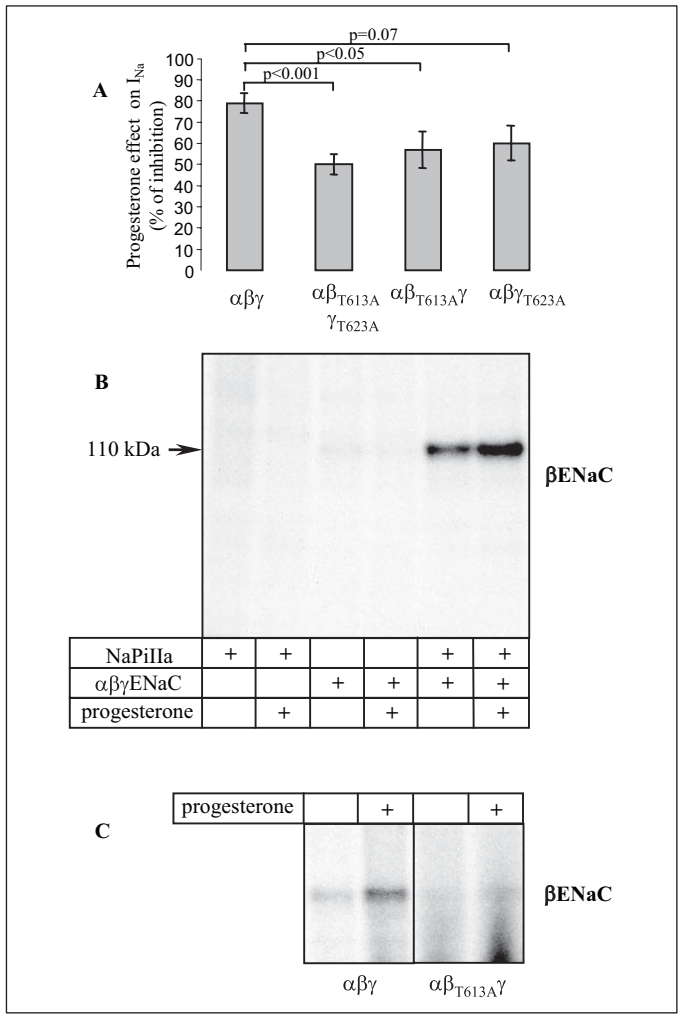

FIGURE 3. **β-Tyr-613 and γ-Tyr-623 residues are involved in the progesterone effect on ENaC.** *A*, expression of β-Tyr-613 (β_{T613A}) or γ-Tyr-623 (γ_{T623A}) mutants results in a significant reduction of the inhibitory effect of progesterone on I_{Na}. Shown are mean ± S.E. of eleven independent 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 $\alpha\beta\gamma$ENaC cRNAs and NaPilla cRNA. 24 h after injection, oocytes were loaded for 4 h with [^{32}P]orthophosphate. Immunoprecipitation was performed in non-denaturing conditions, using anti-FLAG antibody. *C*, progesterone-induced phosphorylation of βENaC is abolished in β_{Y618A} mutant. Oocytes were co-injected with NaPilla cRNA and $\alpha\beta\gamma$ENaC or $\alpha\beta_{Y618A}\gamma$ENaC FLAG-tagged cRNAs. 24 h after injection, oocytes were loaded for 4 h with [^{32}P]orthophosphate. Immunoprecipitation was performed in non-denaturing conditions, using anti-FLAG antibody.

mutant (Nedd4-2CS mutant) on channel activity. As shown in Fig. 2*C*, co-expression of ENaC with the Nedd4-2CS mutant leads to a complete loss of the progesterone effect. As expected, PY mutations (Fig. 2*B*) or co-expression with the Nedd4-2CS mutant (Fig. 2*C*) 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 Nedd4-2 (9, 14). For instance, Shi *et al.* (9) have shown that phosphorylation of βThr-613 and γThr-623 by ERK facilitates 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 phosphorylation sites on progesterone inhibition of I_{Na}. As shown in Fig. 3*A*, the mutation of βThr-613 and/or γThr-623 to alanine results in a partial but significant reduction of the progesterone effect on I_{Na}. These mutations also resulted in a significant increase in basal I_{Na}: 8.8 ± 2.3-fold increase for $\alpha\beta_{T613A}\gamma_{T623A}$ channel ($p < 0.01$, compared with $\alpha\beta\gamma$ channel); 2.7 ± 0.6-fold increase for $\alpha\beta_{T613A}\gamma$ channel ($p < 0.05$, compared with $\alpha\beta\gamma$ channel); and 2.5 ± 0.8-fold increase for $\alpha\beta_{T613A}\gamma_{T623A}$ channel ($p < 0.05$, compared with $\alpha\beta\gamma$ channel). To check whether progesterone induces ERK-dependent ENaC phosphorylation, we attempted ^{32}P-phosphorylation experiments in living oocytes. However, our first experiments revealed only low, almost undetectable levels of ENaC phosphorylation (data not shown). This was probably due to a low uptake of [^{32}P]orthophosphate across the oocyte plasma membrane under standard conditions. To improve oocyte loading with [^{32}P]orthophosphate, we co-injected ENaC subunits with NaPiIIa sodium phosphate co-transporter. As shown in Fig. 3*B*, co-injection

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. 3*C*). 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 wcP_o (10). The effect of progesterone on wcP_o 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 (γ_{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. 4*A*, 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 the $\alpha\beta\gamma_{Y628A}$ channel, whereas the intensity of the ~65-kDa band remained unchanged. In the intracellular protein pool, the anti-α antibody specifically 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. 4*D*). 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. 4*B*, the anti-β antibody specifically recognizes two molecular species of βENaC expressed at the cell surface: a major band

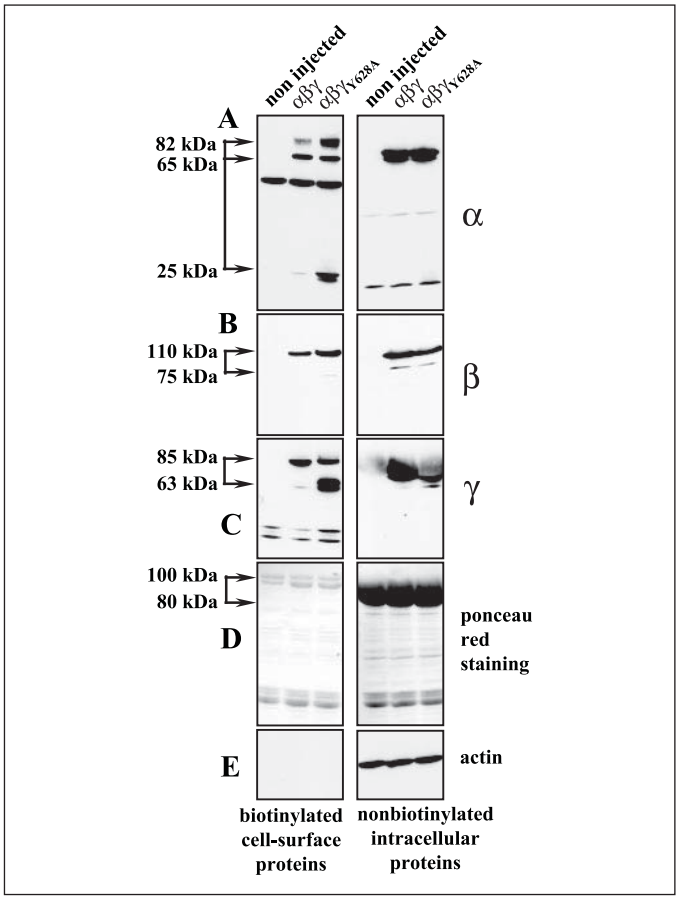

FIGURE 4. **Cell surface biotinylation of ENaC subunits.** *A*, cell-surface biotinylated proteins (*left panel*) and an intracellular protein pool (*right panel*) were probed with an anti-αENaC antibody. This antibody recognizes an N-terminal epitope in the αENaC subunit. *B*, cell-surface biotinylated proteins (*left panel*) and an intracellular protein pool (*right panel*) were probed with an anti-βENaC antibody. This antibody recognizes a 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.

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 $\alpha\beta\gamma_{Y628A}$ 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. 4*C*, 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 $\alpha\beta\gamma_{Y628A}$ 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. 4*E*). The absence of the 25-kDa

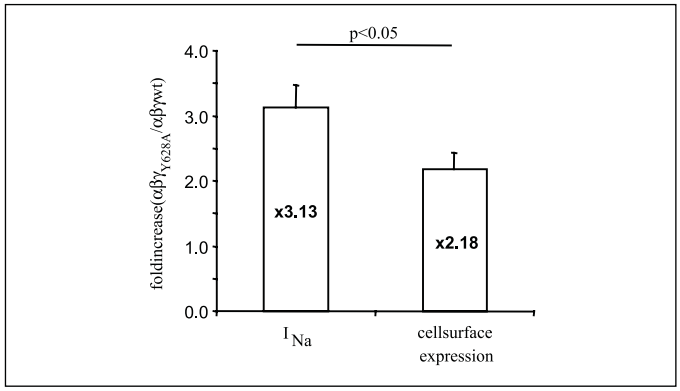

FIGURE 5. **Dual effect of the γY628A mutation on *N* and P_o.** Expression of $\alpha\beta\gamma_{Y628A}$ results in a 3.13 ± 0.35-fold increase in I_{Na}, whereas the parallel quantification of Western blots revealed only a 2.18 ± 0.25 increase in βENaC cell-surface expression. Shown are mean ± S.E. of twenty-five independent experiments each performed with five oocytes per experimental condition. *, statistical significance of <0.05.

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 singe major band of ~95 kDa. As shown in Fig. 5, the $\alpha\beta\gamma_{Y628A}$ channel exhibits 3.13-fold increased I_{Na}, 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_o (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_o—To assess the mechanism of the progesterone effect on ENaC activity, we performed a parallel analysis of I_{Na} and cell-surface expression of ENaC in oocytes treated with progesterone. As shown in Fig. 6*A*, 4-h treatment with progesterone resulted in a ~80% inhibition of I_{Na}. Biotinylation experiments presented in Fig. 6*B* 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. 6*C*). 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 wcP_o.

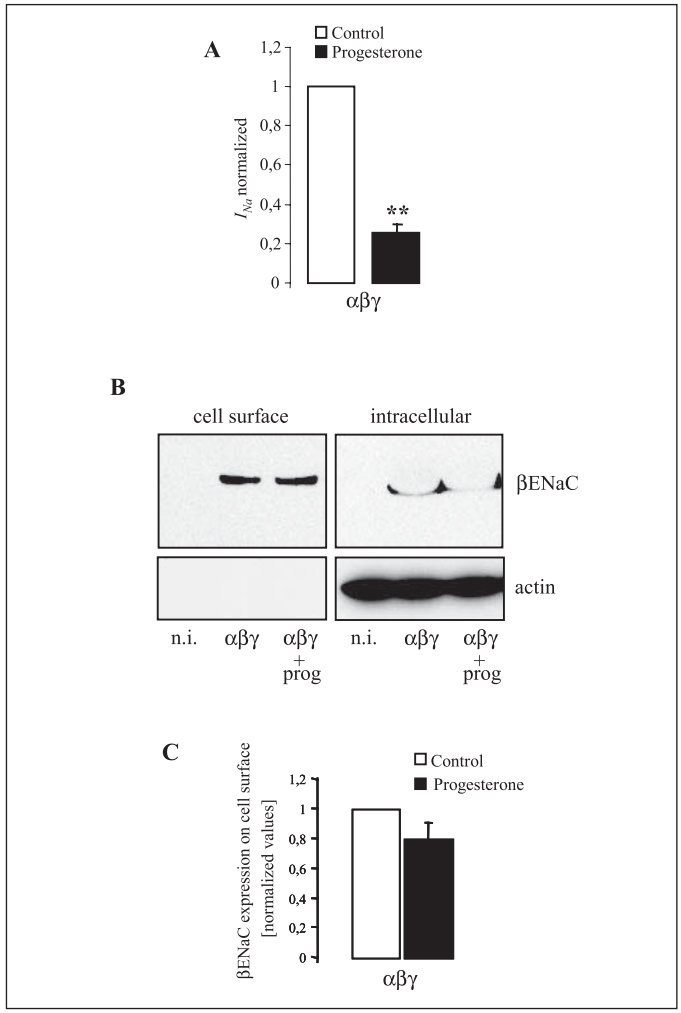

FIGURE 6. **Progesterone down-regulates ENaC P_o.** *A*, progesterone significantly down-regulates I_{Na}. Oocytes were injected with αβγENaC cRNAs. After injection, the oocytes were incubated in MBS solution for 24 h. Oocytes were treated for 4 h with progesterone (15 mM) before I_{Na} measurement. Shown are mean ± S.E. of nine independent experiments each performed with five oocytes per experimental condition. *, statistical significance of <0.001. *B*, Western probing of cell surface (*left panel*) and intracellular proteins (*right panel*) demonstrates that there is no significant change in βENaC expression upon progesterone treatment. *C*, statistical treatment (*t* test) of nine independent biotinylation experiments demonstrated that there is no significant difference in the cell-surface expression of the βENaC subunit between untreated oocytes and oocytes treated with progesterone. Shown are mean ± S.E. of nine independent experiments each performed with 20 oocytes per biotinylation.

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 wcP_o (6). To explain this double effect of PY mutation, one might propose either the existence of a Nedd4-2-inde-

pendent, but PY motif-dependent mechanism, regulating channel P_o, or alternatively, the involvement of Nedd4-2 in regulating both N and P_o.

Our present study favors the latter, showing that Nedd4-2 can be directly involved in the regulation of ENaC P_o. In *Xenopus* oocytes, the activation of the MAP kinase cascade by progesterone leads to the Nedd4-2-dependent decrease in channel wcP_o. Importantly, this effect of progesterone requires both Nedd4-2 binding to ENaC via PY motifs and Nedd4-2 catalytic activity. Because Nedd4-2 binding to PY motifs is also required for regulation of channel N, the difference may arise from the amino acids lying outside of the canonical PY motif involved in ENaC-Nedd4-2 binding. Our data demonstrate that βThr-613 and/or γThr-623 are required for the full effect of progesterone on ENaC. Shi *et al.* (9) have shown that phosphorylation of these amino acids by ERK facilitates ENaC-Nedd4 interaction. One may propose that these additional binding sites may be responsible for the novel effect of Nedd4-2 on channel P_o. Alternatively, progesterone treatment may lead to Nedd4-2-dependent ubiquitylation of non-ENaC proteins. Also, we cannot formally rule out the possibility that progesterone affects the channel conductance, but it is, at present, a very unlikely possibility, because the C terminus of either β or γ ENaC subunit is located far away from the channel pore region. All mutations of the C termini so far studied have changed either N and/or P_o but not channel conductance.

Previously, several lysine residues in N-terminal parts of α and γ ENaC subunits have been identified as the targets for Nedd4-2-dependent ubiquitylation (25). The major effect of the mutation of these lysine residues was an increase of channel N. Whether ubiquitylation of other lysines may influence the mode of Nedd4-2 action on ENaC remains to be established. Recently, Rajan *et al.* (26) have demonstrated that addition of SUMO, a ubiquitin-like protein, to a single lysine residue in the K2P1 (TWIK) potassium channel results in a complete silencing of this channel. Removal of SUMO by a desumoylation enzyme restored channel activity. This study provided the proof of principle that a ubiquitylation-like mechanism can be involved in the regulation of the open probability of ion channels.

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} measured in the principal cell when the animals were fed with a low sodium diet or when they were infused with aldosterone. The I_{Na} from Liddle mice was also larger when animals were pretreated with a high potassium diet, a maneuver that increases plasma aldosterone levels. Noise analysis of channel activity indicated that the open probability was similar in the two genotypes. Thus the increase in whole cell current was attributed to a difference in the density of conducting channels, but as discussed by the authors, their method cannot distinguish between *de novo* insertion of channels into the plasma membrane from activation of near silent channels ($P_o <$ 0.05) already expressed at the surface. In addition, our own measurement of wcP_o (6) suggests that the number of channels that can be measured by noise or patch clamp analysis represents only a minor fraction of ENaCs expressed at the cell surface. Immunolocalization studies also show a translocation of the ENaC subunit at the apical membrane under salt restriction (28). No channel protein could be detected by immunostaining in salt-repleted animals. Again, the apparent lack of channels in the apical membrane of principal cells is probably due to the difficulty to detect by immunostaining a small number of channels, which are postulated to be expressed under a standard salt diet. Indeed, if there was no functional channels under this physiological condition, one should not observe severe, life-threatening, salt-loosing syndromes in pseudo-hypoaldosteronism type 1 patients or in mouse models in which ENaC activity has been drastically reduced or totally abolished (29, 30). The absence of a P_o effect by aldosterone may be methodological rather than biological. The apparent controversy will be, however, difficult to solve as long as the measurement of whole cell P_o will be restricted to large cells (oocytes) and will remain technically not feasible in small somatic cells (CCD principal cells)

Physiological Relevance of ERK-dependent Regulation of ENaC Activity—What could be the physiological relevance of the present data? To our knowledge, under physiological conditions, progesterone does not have any significant effect on wild-type ENaC activity in the distal nephron. However, the ERK-dependent inhibition of ENaC activity is the principal mechanism of the epidermal growth factor-induced inhibition of transepithelial sodium transport in the kidney CCD. Shen and Cotton (7) have shown that acute addition of epidermal growth factor to the basolateral side of the CCD monolayer leads to a rapid ERK1/2 phosphorylation/activation and a parallel inhibition of I_{Na}. This effect was abolished by pretreatment of CCD monolayers with an inhibitor of ERK pathway. Veizis and Cotton (31) have proposed that apical delocalization of the epidermal growth factor receptor, leading to the hyperactivation of ERK1/2 and the sustained inhibition of 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.

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