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In vivo phosphorylation of the epithelial sodium channel

(amiloride/insulin/aldosterone/Madin–Darby canine kidney)

RICHARD A. SHIMKETS*, RICHARD LIFTON*, AND CECILIA M. CANESSA†‡

*Department of Genetics and †Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06520-8026

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ABSTRACT **The activity of the epithelial sodium channel (ENaC) in the distal nephron is regulated by an antidiuretic hormone, aldosterone, and insulin, but the molecular mechanisms that mediate these hormonal effects are mostly unknown. We have investigated whether aldosterone, insulin, or activation of protein kinases has an effect on the phosphorylation of the channel. Experiments were performed in an epithelial cell line generated by stable cotransfection of the three subunits (α, β, and γ) of ENaC. We found that β and γ, but not the α subunit, are phosphorylated in the basal state. Aldosterone, insulin, and protein kinases A and C increased phosphorylation of the β and γ subunits in their carboxyl termini, but none of these agents induced *de novo* phosphorylation of α subunits. Serines and threonines but not tyrosines were found to be phosphorylated. The results suggest that aldosterone, insulin, and protein kinases A and C modulate the activity of ENaC by phosphorylation of the carboxyl termini of the β and γ subunits.**

The activity of epithelial sodium channel (ENaC) in the cortical collecting tubule of the kidney is under tight hormonal control. Aldosterone, insulin, and antidiuretic hormone modify sodium reabsorption by markedly increasing the apical permeability of the distal nephron to sodium. This increase in permeability might be achieved by at least two independent mechanisms: synthesis of new sodium channels and activation of preexisting channels (1).

Previous studies have suggested that protein kinases are involved in the regulation of ENaC activity. For instance, antidiuretic hormone increases the concentration of cAMP that activates protein kinase A (PKA), which then stimulates sodium reabsorption (2, 3), whereas activation of protein kinase C (PKC) by phorbol esters or diacylglycerol inhibits sodium uptake (4). The action of insulin on sodium channels has also been attributed to stimulation of kinases (5, 6). Although there is no doubt that the activation of intracellular kinases can modulate the activity of ENaC, the mechanisms involved have not yet been elucidated; moreover, it is not known whether the sodium channel is a substrate for protein kinases.

In the present study we investigated the effect of hormones and protein kinases on the phosphorylation state of the channel subunits and whether the subunits of ENaC are phosphorylated *in vivo*. Studies of phosphorylation were performed in transfected Madin–Darby canine kidney (MDCK) cell lines generated by stable transfection with the rat α, β, and γ subunits of ENaC. We used metabolic labeling with [^{35}S]methionine and [^{32}P]orthophosphate, immunoprecipitation, and phosphoamino acid mapping to identify the subunits that are phosphorylated and to localize the residues that are modified. The results showed that β and γ but not the α subunit are phosphorylated in the basal state. Aldosterone, insulin, PKA, and PKC increased the level of phosphorylation of both β and γ, but they did not induce *de novo* phosphorylation of the α subunit. All phosphorylated residues were serines and threonines; no evidence of phosphotyrosines was found. All phosphorylated amino acids reside in the carboxyl termini of β and γ. The results suggest that some of the hormonal effects on the activity of ENaC are mediated by phosphorylation of the β and γ subunits.

METHODS

Generation of $\alpha\beta\gamma$ MDCK Cell Lines and Cell Culture Methods. MDCK cells were transfected with the plasmid pJB20-αENaC by using liposomes (Lipofectamine). Transformants were selected for resistance to neomycin (Geneticin, GIBCO/BRL) and screened for expression of α subunit by immunoprecipitation with a specific antibody. A cell line expressing α subunits was subsequently cotransfected with the plasmids pCB7-βENaC or pCB7-β_TENaC (β truncated at amino acid R564) and plasmid pCDNA1-γENaC, all under the cytomegalovirus promoter and with the gene conferring resistance to hygromycin B. Colonies resistant to neomycin and hygromycin were screened with β and γ subunit-specific antibodies. Cell lines expressing all three subunits were selected for further studies. Cells were maintained in DMEM supplemented with 10% fetal bovine serum/200 μg/ml neomycin/200 μg/ml hygromycin B.

Measurements of Short Circuit Current (I_{sc}). Wild-type and $\alpha\beta\gamma$ MDCK cells were grown on permeable supports (24 mm diameter, Transwell, Costar) for 10 days. Confluent monolayers were mounted in a modified Ussing chamber (Trans-24 miniperfusion chamber, WPI Instruments, Waltham, MA). Apical and basolateral chambers were continuously bathed with DMEM medium. I_{sc} was measured with transepithelial voltage clamped at 0 mV with a DVC-1000 dual voltage clamp (WPI Instruments). Voltage pulses (10 mV) were applied every 3 min to monitor the transepithelial resistance. After the initial measurements, 10 μM amiloride was added to the apical side, and sodium current was expressed as the amiloride-sensitive component of the I_{sc}.

Metabolic Labeling and Immunoprecipitations. Cells were labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine (67 μCi/ml, Amersham) for at least 5 h in DMEM lacking cysteine and methionine. Cells were washed three times with PBS and lysed with ice-cold homogenization buffer (100 mM NaCl/20 mM Tris·HCl, pH 7.6/1% Triton X-100) containing 2 mM phenylmethylsulfonyl fluoride and 5 μg each of pepstatin, leupeptin, and aprotinin. Debris was removed by centrifugation at 10,000 rpm in a microcentrifuge for 10 min at 4°C, and the supernatant was brought to a final concentration of 3.4%

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Abbreviations: ENaC, epithelial sodium channel; PKA, protein kinase A; PKC, protein kinase C; MDCK, Madin–Darby canine kidney; PMA, phorbol 12-myristate 13-acetate.

‡To whom reprint requests should be addressed at: Dept. of Cellular and Molecular Physiology, 333 Cedar St., New Haven, CT 06520-8026. e-mail: Cecilia.Canessa@Yale.EDU.

SDS and heated at 95°C for 5 min. One milliliter of 1% TENT (50 mM Tris·HCl, pH 7.5/5 mM EDTA/150 mM NaCl/1% Triton X-100) was added, followed by 10 μl of one of the specific anti-α, -β and -γ sera, and the solution was incubated at room temperature for 4 h. The specificity of these antibodies has been previously characterized (7). Sodium orthovanadate (0.2 mM) was added to samples containing antiphosphotyrosine antibodies (Upstate Biotechnology, Lake Placid, NY). After incubation, 50 μl of a 50% slurry of Protein A/Sepharose CL-4B was added, and the samples were rocked for 1 h at room temperature. Beads were washed three times with 1% TENT. Proteins were eluted in 70 μl of Laemmli sample buffer. Immunoprecipitates were electrophoresed on 10% SDS/polyacrylamide gels, stained with Coomassie blue, fixed, treated for 30 min with 1 M salicylic acid, dried, and exposed to Kodak film X-Omat AR at −70°C.

Phosphorylation Experiments. Plates of confluent $\alpha\beta\gamma$ MDCK cells were grown on serum-free medium for 24 h, and then 10^{-6} M aldosterone was added for 0, 3, or 16 h. Subsequently, cells were washed with phosphate-free DMEM and incubated for 3 h in 1 mCi/ml [^{32}P]orthophosphate in the presence or absence of aldosterone. In separate experiments, cells were stimulated for 15 min with 10 nM insulin (Calbiochem), 100 mM phorbol 12-myristate 13-acetate (PMA), or 50 μM forskolin plus 5 mM 3-isobutylmethylxanthine. Channel subunits were immunoprecipitated as above, but the lysis buffer was supplemented with phosphatase inhibitors: 0.2 mM sodium orthovanadate, 50 mM sodium fluoride, and 30 mM sodium pyrophosphate. After electrophoresis, proteins were transferred to nitrocellulose membranes by electroblotting. Membranes were exposed to film at −70°C with an intensifying screen. Autoradiographs from phosphorylated subunits were quantified by scanning densitometry with a ScanMaker scanner or PhosphorImager (Molecular Dynamics).

Phosphoamino Acid Analysis. Phosphoamino acid maps of phosphorylated β and γ subunits were generated by excising the appropriate bands from the nitrocellulose membranes and eluting the proteins with pyridine at 37°C for 30 min. Radioactivity incorporation was determined by Cerenkov counting. The samples were lyophilized to dryness and dissolved in 100 μl of constant boiling 6 M HCl and hydrolyzed at 110°C for 1 h in a vacuum oven. The samples were again lyophilized, dissolved in running buffer, and spotted onto thin-layer cellulose plates together with 10 μg each of the phosphoamino acid standards phosphoserine, phosphothreonine, and phosphotyrosine. Phosphoamino acids were separated by two-dimensional electrophoresis (2.5 kV), first in formic acid (4.4% formic acid/15.5% glacial acetic acid, pH 1.9) for 15 min and subsequently in acetic acid buffer (10% glacial acetic acid/1% pyridine, pH 3.5) at 2.0 kV for 10 min. Plates were dried at 65°C and sprayed with ninhydrin (0.2% in acetone) to reveal the phosphoamino acid standards. Plates were then exposed to film at −70°C (8).

Phosphopeptide Mapping. Two-dimensional phosphopeptide maps were generated by treating excised Immobilon-P bands containing immunopurified phosphorylated β subunit with 0.5% polyvinylpyrrolidone in 100 mM acetic acid for 30 min at 37°C, followed by 5 rinses in deionized water and 2 rinses in 0.05 M ammonium bicarbonate. The membrane-bound protein was then digested in 200 ml of 0.05 M ammonium bicarbonate by 20 mg of chymotrypsin for 2 h at 37°C. Following digestion and lyophilization, samples were oxidized on ice in 50 ml of performic acid (formic acid:hydrogen peroxide, 9:1). Samples were lyophilized and then spotted onto thin-layer cellulose plates. Plates were electrophoresed for 25 min at 1.2 kV in ammonium carbonate at pH 8.9. After drying, plates were turned 90° and subjected to ascending chromatography in phosphochromatography buffer (*n*-butyl alcohol:pyridine:glacial acetic acid:deionized water, 15:10:3:12). After drying, plates were exposed to film at −70°C or to a phosphorimaging screen (8).

RESULTS

Generation of $\alpha\beta\gamma$ MDCK Cells and Response to Agonists. To investigate the phosphorylation state of ENaC we generated a stable cell line that expresses high levels of the three subunits of the channel by transfecting MDCK cells with cDNAs of α, β, and γ subunits. As shown in Fig. 1, the levels of expression of the three subunits were robust and approximately equal as demonstrated by immunoprecipitation of metabolically steady-state labeled cells with [^{35}S]methionine. The antibodies did not precipitate proteins in the wild-type MDCK cell line, indicating that all the bands shown in the experiments correspond to the transfected subunits.

Expression of functional channels was examined by measuring the I_{sc} of cells grown on permeable supports for 10 days. The transepithelial voltage and the resistance of transfected cells were 4 ± 1.8 mV and 60 ± 22 ohm·cm^2, respectively. Basal I_{sc} was 5 ± 1.7 μA/cm^2 compared with the value of 0.2 ± 0.8 μA/cm^2 in the parental cell line.

The effects of aldosterone, insulin, PMA, and forskolin on the amiloride-sensitive component of the I_{sc} were evaluated. To examine the long-term effects of aldosterone, cells were deprived of serum for 24 h. Aldosterone (1 μM) was added to the basolateral side 3 or 24 h before experiments. Cells treated with aldosterone exhibited 40% higher amiloride-sensitive I_{sc} than controls (Table 1). Insulin applied to the basolateral side induced a rapid response with a 48% increase in I_{sc}. Forskolin increased the I_{sc} by 80%. However, 30% of the forskolin-induced increase in I_{sc} was not amiloride-sensitive. The amiloride-insensitive component was attenuated in chloride-free solutions, indicating that it was probably mediated by chloride secretion. Addition of PMA caused a marked decrease in the transepithelial resistance, and the I_{sc} became unstable, making it difficult to evaluate the effect on the amiloride-sensitive current. Fig. 2 shows a tracing of I_{sc} before and after adding 10 μM amiloride to the apical side and the increase of current induced by 50 μM forskolin.

Basal Phosphorylation of ENaC and Response to Aldosterone, Insulin, and Activation of Protein Kinases. The basal phosphorylation state of ENaC was examined by maintaining the $\alpha\beta\gamma$ MDCK cells in serum-free medium for at least 12 h before experiments. The cells were then labeled with ^{32}P, and

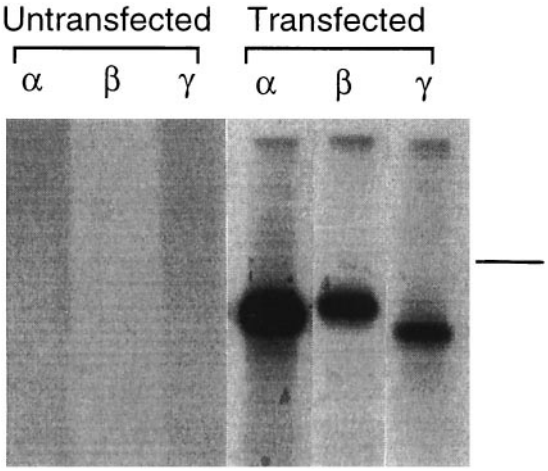

FIG. 1. Expression of the three subunits of ENaC in a stably transfected MDCK cell line. Wild-type (lanes 1–3) and $\alpha\beta\gamma$-MDCK cells (lanes 4–6) were labeled with [^{35}S]methionine, followed by immunoprecipitations with specific anti-α (lanes 1 and 4), anti-β (lanes 2 and 5), and anti-γ (lanes 3 and 6) antibodies. Bar indicates molecular mass of 85 kDa.

Table 1. Amiloride-sensitive I_{sc} of $\alpha\beta\gamma$ MDCK cells under control and stimulated conditions

Treatment	I_{sc}, $\mu A/cm^2$
Control	4.8 ± 1.3
Aldosterone (3 h)	9.5 ± 1.2
Aldosterone (24 h)	8.9 ± 1.5
Insulin (15 min)	8.5 ± 1.1
Forskolin (15 min)	10.5 ± 2.9

the subunits of the channel were recovered by immunoprecipitation as described under *Methods*. Fig. 3 shows that under control conditions, both β and γ subunits are phosphorylated, whereas the α subunit is not phosphorylated.

The effect of aldosterone was examined on $\alpha\beta\gamma$ MDCK cells deprived of serum for 24 h and then treated with aldosterone for 0, 3, or 16 h. During the last 3 h before immunoprecipitation, cells were incubated in [^{32}P]orthophosphate. Fig. 3 shows that aldosterone induced a significant increase in the level of phosphorylation of the β and γ subunits at the 3- and 16-h time points. In contrast, α subunit was not phosphorylated. The increased signal was not because of a change in channel abundance, because there was no significant difference in the total amount of protein recovered in the presence and absence of aldosterone. These results suggest a novel mechanism of aldosterone action and are consistent with the view that aldosterone changes the activity of the channel by posttranslational modification of existing channels. The finding that the phosphorylation is evident as early as 3 h after addition of the hormone further suggests that it may play a role in mediating the early effects of aldosterone on the ENaC (9).

The effects of insulin and of activating PKC and PKA on the phosphorylation of ENaC were examined on $\alpha\beta\gamma$ MDCK cells labeled with ^{32}P and treated with either insulin, PMA, or forskolin for 15 min. Analysis of the immunoprecipitated subunits revealed that the β and γ subunits had approximately 10- and 5-fold increases, respectively, in the intensity of phosphorylation. In contrast, the α subunit was not phosphorylated (Fig. 3, lane 4). Addition of a permeant analog of cGMP did not induce phosphorylation of ENaC (data not shown).

Residues Phosphorylated in the β and γ Subunits. To determine the residues that are phosphorylated we performed phosphoamino acid analysis of ^{32}P-labeled β and γ subunits in untreated cells and in cells treated with aldosterone for 24 h. Phosphoamino acid mapping of the β subunit of nonstimulated cells showed only phosphoserine residues (Fig. 4*A*), whereas in aldosterone-treated cells the β subunit showed stronger phosphoserine and a new phosphothreonine signal (Fig. 4*B*). Under the same conditions, in both control and aldosterone-treated

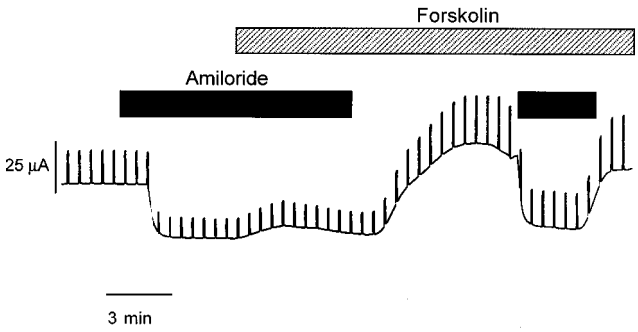

FIG. 2. Effects of amiloride and forskolin on the I_{sc} of $\alpha\beta\gamma$ MDCK cells. Tracing shows inhibition of I_{sc} of $\alpha\beta\gamma$ MDCK cells by 5 μM apical amiloride. Forskolin stimulated amiloride-insensitive and -sensitive components of the I_{sc} and increased the conductance indicated by the height of the spikes in the current tracing. Transepithelial voltage was clamped at 0 mV; 10-mV pulses were given at intervals of 3 min.

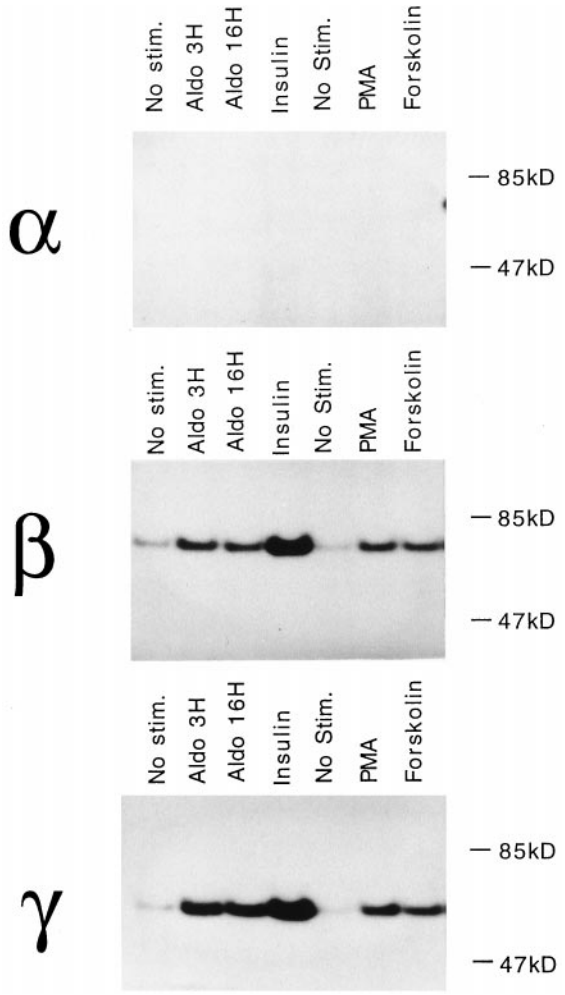

FIG. 3. Phosphorylation of ENaC after stimulation by aldosterone, insulin, and activation of kinases. $\alpha\beta\gamma$ MDCK cells were labeled with ^{32}P followed by no treatment, 3 and 16 h of aldosterone (Aldo), or 15 min of insulin, PMA, or forskolin/3-isobutylmethylxanthine. α, β, and γ subunits were immunoprecipitated from equal amounts of cell lysates, and the products were resolved by SDS electrophoresis.

cells, the γ subunit showed phosphoserine and phosphothreonine in approximately equal amounts (Fig. 4 *C* and *D*).

Although tyrosine phosphorylation was not detected in the phosphoamino acid analyses, the partial acid hydrolysis used to isolate the phosphoamino acids biases against the recovery of phosphotyrosine. We used monoclonal antiphosphotyrosine antibodies in Western blots and in immunoprecipitations as alternative approaches to determine tyrosine phosphorylation of the channel subunits. $\alpha\beta\gamma$ MDCK cells were nonstimulated, treated with 10 nM insulin, or with 1 μM of aldosterone for 24 h followed by immunoprecipitations with anti-ENaC sera and subsequently with antiphosphotyrosine antibody. Fig. 5 shows that there was no tyrosine phosphorylation in control, in aldosterone, or in insulin-stimulated cells (lanes 1, 5, and 9). Lane 14 shows that the antiphosphotyrosine antibody immunoprecipitates many other proteins that were unrelated to the channel subunits. Lanes in Fig. 5 show α, β, and γ subunits recovered after the antiphosphotyrosine immunoprecipitation to demonstrate the presence of subunits in the samples. In Western blotting analysis the antiphosphotyrosine antibody also gave negative results (data not shown).

Localization of the Phosphorylated Residues in the β Subunit. According to the membrane topology of ENaC, the amino and carboxyl termini are the only cytoplasmic domains of the subunits (10, 11) and, therefore, the only regions where

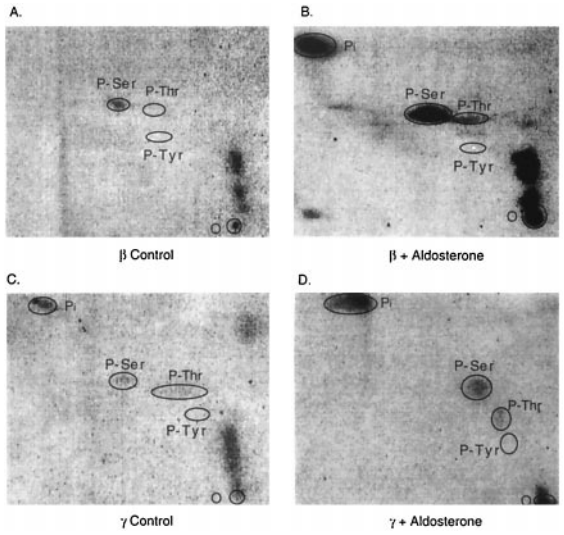

FIG. 4. Phosphoamino acid analysis of β and γ subunits of the ENaC without and with aldosterone. $\alpha\beta\gamma$ MDCK cells were grown in serum-free medium for 24 h before experiments. Cells were then treated with aldosterone for 16 h. During the last 3 h of incubation, 1 mCi/ml ^{32}P was added to the medium. β and γ subunits were isolated by immunoprecipitation followed by SDS/gel electrophoresis. Acid hydrolysates of the proteins were spotted onto cellulose plates and separated by thin-layer electrophoresis in two dimensions. Middle circles indicate migration positions of phosphorylated standards; O is the origin and P_i is the completely hydrolyzed phosphate.

phosphorylation can take place *in vivo*. To determine the location of the phosphorylated amino acids in the β subunit we generated a mutant form βR564stop (β_T) that deletes all the carboxyl terminus of the protein and cotransfect it with wild-type α and γ subunits into MDCK cells. Fig. 6 shows the level of expression of the transfected wild-type β and β_T subunits evaluated by immunoprecipitation after labeling the cells with [^{35}S]methionine. Phosphorylation experiments performed in these cells revealed that β_T did not incorporate ^{32}P, indicating that the phosphorylated residues are located in the carboxyl terminus.

To define better the location of the phosphorylated residues we performed phosphopeptide analysis by two-dimensional

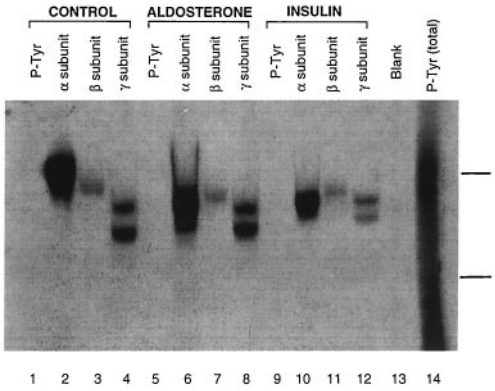

FIG. 5. Absence of phosphotyrosines (P-Tyr) in the channel subunits. $\alpha\beta\gamma$ MDCK cells were labeled with [^{35}S]methionine and treated either with aldosterone for 16 h or with insulin for 10 min. The subunits were recovered by anti-α, -β, and -γ sera. The products of the first immunoprecipitation were subjected to a second round of immunoprecipitation with an antiphosphotyrosine antibody (lanes 1, 5, and 9). The channel subunits not bound by the antiphosphotyrosine antibody were reprecipitated with specific subunit antibodies (lanes 2–4, 6–8, and 10–12). Lane 14 shows all proteins immunoprecipitated with the antiphosphotyrosine antibody from a lysate of insulin-stimulated cells. Bars indicate molecular masses of 85 and 45 kDa, respectively.

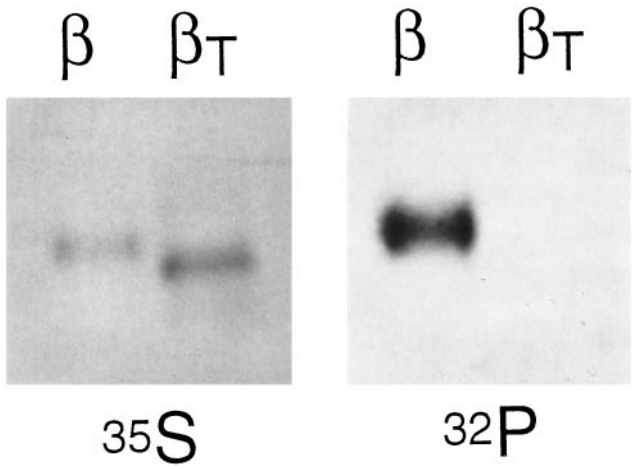

FIG. 6. Phosphorylation of wild-type and β_T subunits. Stably transfected MDCK cells expressing $\alpha\beta\gamma$ and $\alpha\beta_T\gamma$ subunits were labeled with [^{35}S]methionine or with [^{32}P]orthophosphate. β subunits were immunoprecipitated with a specific antibody against the amino terminus of the protein.

separation on thin-layer cellulose plates. $\alpha\beta\gamma$ MDCK cells were labeled with ^{32}P without or with treatment with aldosterone or insulin. The carboxyl terminus of the β subunit contains chymotryptic sites at positions Y^{569}, F^{588}, Y^{602}, and Y^{618}. Digestion with chymotrypsin generates four peptides (Fig. 7*D*). In the nonstimulated state only one phosphopeptide is seen; however, after stimulation with aldosterone or insulin two new signals appeared, indicating that these hormones induced phosphorylation of additional residues. According to the predicted mobility of the chymotryptic peptides (8), the signal shown in unstimulated cells may correspond to peptide 4, which after stimulation appears as a doublet, consistent with the incorporation of another phosphate to the same peptide. Aldosterone and insulin also induced a third phosphorylated signal that may correspond to peptides 1 or 3.

DISCUSSION

This study demonstrates *in vivo* phosphorylation of ENaC and that aldosterone and insulin, two of the most important

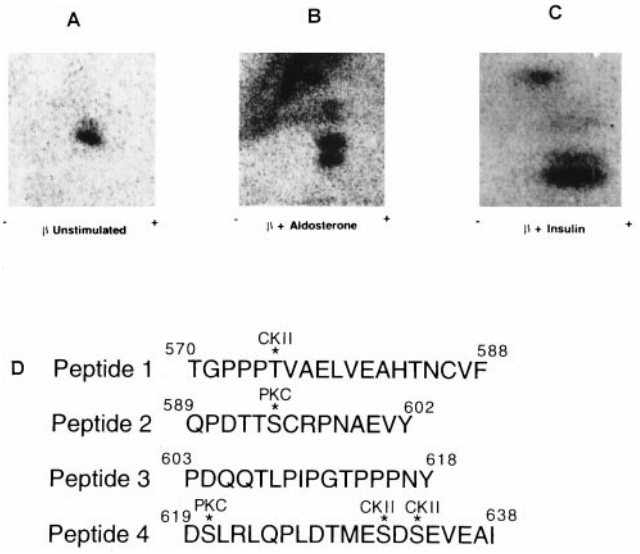

FIG. 7. Phosphopeptide analysis of the β subunit. Immunoprecipitated β subunit was digested with chymotrypsin, resolved in two dimensions on TLC plates, and visualized by autoradiography. (*A*) Nonstimulated cells. (*B*) Aldosterone for 3 h. (*C*) Insulin. (*D*) Peptides generated by chymotrypsin digestion of the carboxyl terminus of the β subunit.

hormonal regulators of sodium channel activity, modify the level of phosphorylation of the channel.

Phosphorylation of ENaC occurs only in the carboxyl termini of the of the β and γ subunits but not in the α subunit. It has been shown that the carboxyl-terminal domains of β and γ regulate the activity of ENaC (12) by the association with Nedd4 (a ubiquitin ligase) (13) and with proteins of the endocytic machinery (14). In particular, tyrosines αY^{673}, βY^{618}, and γY^{628} have been shown to be essential in the interaction with the regulatory proteins (15, 16), raising the possibility that phosphorylation of these tyrosines could provide a mechanism for modulating the interactions with these proteins. The absence of phosphotyrosines in the subunits of ENaC did not confirm this hypothesis. However, the presence of phosphorylated serines and threonines in the vicinity suggests that these other residues could modulate protein interactions with the carboxyl termini. Alternatively, phosphorylation could serve as a signal for ubiquitination of the channel by Nedd4 as has been demonstrated for IκB-α (17) and β-catenin (18).

The finding of phosphorylated channels in nonstimulated cells may indicate that tonic activity of kinases is necessary to maintain basal sodium transport, and is consistent with electrophysiological studies reporting rundown of channel activity in excised patches, probably because of the inability of the channel to maintain its phosphorylated state (19). Alternatively, basal phosphorylation may serve to down regulate basal transport chronically.

Previous reports are consistent with the notion that PKA augments the activity of ENaC by increasing the density of active channels at the plasma membrane (20, 21) either by incorporating channels from a vesicular pool or by activating channels at the cell surface. Because the effects of PKA are tissue-specific a direct effect of PKA on channel kinetics is unlikely. Instead phosphorylation of ENaC might be necessary for the action of the tissue-specific proteins that mediate trafficking of channels. In contrast, PKC, when applied to cells (22–24) or to reconstituted channels in lipid bilayers (25), reduces channel activity by decreasing open probability (P_o), suggesting that phosphorylation affects channel kinetics. However, our results cannot explain the decrease in P_o that was observed in reconstituted channels formed only by α subunits (25), because we did not detect phosphorylation of α subunits under any conditions.

Aldosterone produced an increase in the level of phosphorylation of β and γ subunits by inducing phosphorylation of new serine and threonine residues. The phosphoamino map and the phosphopeptide analysis suggest that at least two new residues in the carboxyl-terminal domain of the β subunit are phosphorylated on stimulation with aldosterone: a threonine residue in peptide 1 and a new serine or threonine in peptide 4. The effects of aldosterone were independent of transcription of new subunits because in the system used in this study transcription of ENaC was under control of the cytomegalovirus promoter that does not respond to steroids. However, the $\alpha\beta\gamma$ MDCK-transfected cells are sensitive to mineralocorticoids (26, 27), which, therefore, are able to activate the pathways that lead to modification of the channel subunits.

The different time courses of the aldosterone and insulin responses suggest that these hormones activate different cellular processes that ultimately phosphorylate the subunits of ENaC. The increase in phosphorylation induced by aldosterone was seen at 3 h and persisted during the 24-h period of stimulation. The response to insulin was evident after 15 min. The finding that aldosterone modifies channels during the early response period suggests that phosphorylation could mediate the activation of preexisting channels.

The results demonstrate that ENaC is phosphorylated by several stimuli and suggest that phosphorylation may mediate some of the effects of hormones and kinases on the activity of the channel. However, the data do not indicate the mechanism by which phosphorylation changes channel activity. The main possibilities to be investigated are changes in kinetics and cellular redistribution of channels.

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