Carboxylmethylation of the β Subunit of xENaC Regulates Channel Activity*

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The action of aldosterone to increase apical membrane permeability in responsive epithelia is thought to be due to activation of sodium channels. Aldosterone stimulates methylation of a 95-kDa protein in apical membrane of A6 cells, and we have previously shown that methylation of a 95-kDa protein in the immunopurified Na⁺ channel complex increases open probability of these channels in planar lipid bilayers. We report here that aldosterone stimulates carboxylmethylation of the β subunit of xENaC in A6 cells. *In vitro* translated β subunit, but not α or γ, serves as a substrate for carboxylmethylation. Carboxylmethylation of ENaC reconstituted in planar lipid bilayers leads to an increase in open probability only when β subunit is present. When the channel complex is immunoprecipitated from A6 cells and analyzed by Western blot with antibodies to the three subunits of xENaC, all three subunits are recognized as constituents of the complex. The results suggest that Na⁺ channel activity in A6 cells is regulated, in part, by carboxylmethylation of the β subunit of xENaC.

Aldosterone-stimulated sodium channel activity has been shown to involve methylation of membrane proteins (1, 2). Moreover, the aldosterone-induced increase in the activity of xENaC is blockable by the methylation inhibitor, 3-deazaadenosine (3). Sariban-Sohraby has demonstrated that aldosterone stimulates the methylation of a 90–95-kDa protein in the apical membrane of A6 cells (4). We have previously shown that methylation of the 95-kDa subunit of an immunopurified renal sodium channel complex reconstituted in lipid bilayers results in increased sodium channel activity (5). In both studies the identity of the methylated protein is unknown.

The epithelial Na⁺ channel from A6 cells (xENaC) has recently been cloned and is made up of three homologous subunits, α, β, and γ (6, 7). When all three subunits are expressed in a *Xenopus* oocyte expression system, a low conductance, Na⁺-selective, amiloride-sensitive Na⁺ channel typical of native cortical collecting duct is observed (7). Although expression of the α subunit is sufficient to generate Na⁺ channel activity, co-expression of all three subunits is necessary for maximal activity (7). By contrast, when β and γ are expressed individ-

ually or together, no Na⁺ channel activity is detectable (6, 7, 8). Thus β and γ subunits of xENaC demonstrate an important role in the regulation of Na⁺ channel activity. The observation that COOH-terminal truncations in either the β or γ subunit are responsible for the Na⁺ channel hyperactivity in Liddle's syndrome is also consistent with a regulatory role for these subunits (9, 10).

Because the glycosylated forms of β- or γ-xENaC migrate as 97-kDa proteins (11–13), we tested the hypotheses that these channel components are the substrates for methylation and that methylation of either or both of these channel components results in increased activity of the xENaC channel.

EXPERIMENTAL PROCEDURES

Culture of A6 Cells and I_{sc} Measurements—Cells were grown as described previously in amphibian medium with 10% fetal bovine serum in an atmosphere of humidified air-4%CO_2 at room temperature (14). To ensure high rates of basal transport, cells from American Type Culture Collection have been cloned by limiting dilution and selected for high amiloride-sensitive I_{sc}. Cells were grown on millicell inserts (Millipore, Bedford, MA). Transepithelial potential difference, resistance, and I_{sc} were measured on each as described previously (14).

Generation of Antibodies—We synthesized peptides with the amino acid sequence corresponding to the carboxyl-terminal regions of the translated protein with an amino-terminal cysteine: α, NH₂-CESLD-LRSVGTLSSRSSSMRSNRSYYEENGGRRN-COOH; β, NH₂-CDFDH-VPVDIPGTPPPNYDSLRVNTAEPVSSDEEN-COOH; and γ, NH₂-CG-EEDPPTFNSALQLPQSQDSHVPRTPPPKYNTLRIQSAFGLETIDSDE DVERL-COOH. Each peptide was linked to Keyhole limpet hemocyanin. The α and γ peptides were injected into chickens and boosted at 1, 2, and 3 months. The β peptide was injected into rabbits and boosted on the same schedule. Antibodies from rabbit serum were immunopurified using a peptide affinity column as described (15). Antibodies from chicken yolk were isolated using a gamma Yolk purification kit (Amersham Pharmacia Biotech) according to kit instructions. The resulting suspension was then passed through the peptide column and isolated as described above.

In Vitro Expression of Full-length xENaC—Full-length α, β, and γ xENaC were subcloned behind the T7 promoter for use in an *in vitro* translations. The α, β, and γ xENaC subunits used in the methylation reactions were generated using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI). The product was labeled by the addition of [³⁵S]cysteine (NEN Life Science Products) at a final concentration of 0.3 mCi/ml. Labeled proteins were subjected to SDS-PAGE¹ to ensure proper size.

Apical Membrane Preparations—A6 cells were grown on 25-mm 0.02 μM Anopore membrane tissue culture inserts (Nalge Nunc Int., Naperville, IL) and pre-treated as necessary prior to the biotinylation procedure. Apical membrane preparations were prepared as described previously (16). For each sample, 150 μg of protein incubated with prewashed avidin beads (Pierce) at 4 °C overnight. Beads were collected

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¹ The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; MOPS, 4-morpholinepropanesulfonic acid.

and washed in NET buffer (0.1% (w/v) SDS, 2 mM EGTA, 10 mM Tris-HCl, pH 7.4). Bound proteins were eluted using SDS-PAGE sample buffer. SDS-PAGE was carried out on 10% SDS-polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes and subjected to Western blotting.

Western Blotting—This was performed as described previously (14). Reactive proteins were detected using enhanced chemiluminescence system (Pierce, ULTRA-ECL) followed by autoradiography.

Immunoprecipitation—This was performed as described previously (14). For immunoprecipitation with chicken antibodies, immobilized anti-chicken IgY (Promega, catalog number G1191) was used in place of Gamma-bind Sepharose beads.

Preparation of Carboxylmethyltransferase—The enzyme was prepared from A6 cells grown on plastic dishes. The cells were disrupted by freeze-thawing and subjected to a $5000 \times g$ spin for 10 min to remove undisrupted cells. The resulting supernatant was centrifuged at $100,000 \times g$ for 1 h. The pellet was resuspended in homogenization buffer (100 mM Tris-HCl, pH 7.4, 1 mM DTT, and protease inhibitor mixture). Enzyme activity was determined by incubating 50 μl of this preparation in the presence or absence of 100 μM N-acetyl farnesyl cysteine, a synthetic substrate for carboxylmethylation (17) in the presence of [^{3}H]AdoMet (10 μl of [^{3}H]adenosyl-L-methionine; 55–85 Ci/mmol, NEN Life Science Products). Enzyme activity was measured by the method of Volker *et al.* (18) and others (15) and was 2.46 ± 0.09 pmols/μg protein/30 min in the presence of GTP.

In Vitro Methylation of A6 Membranes—A6 cells were grown to confluence and then exposed to 1 μM aldosterone or diluent for 4 h. Transepithelial short circuit current was measured prior to and 4 h after addition of aldosterone to document hormone responsiveness and cell viability (data not shown). Cells were then scraped into homogenization buffer and disrupted by repeated freeze-thaw. Membranes were isolated by centrifugation as above. Protein-matched samples were incubated with [^{3}H]AdoMet in the presence or absence of 100 μM GTPγS for 1 h at 27 °C. The reaction was quenched by the addition of 4-fold concentrated sample buffer, and the proteins were resolved by SDS-PAGE and detected by autoradiography.

Determination of Base-labile Counts—To determine whether proteins were carboxylmethylated, the vapor phase method of Clarke *et al.* (19) was employed. Proteins from either whole cell lysates or methylated cell membrane immunoprecipitation with either the β or γ-xENaC antibody were separated by SDS-PAGE. Gels were dried and sliced into 1-mm sections from the top of the lane to the dye front. The slices were placed into open ended Eppendorf tubes and exposed to 1 M NaOH. The Eppendorf tubes were then carefully lowered into sealed scintillation vials containing 10 ml of liquid scintillation fluid. After a 24-h incubation at 37 °C, the Eppendorf tubes were removed. The radioactive methanol released as a result of base-mediated breakage of carboxylmethyl esters was counted. These base labile counts are a direct measure of protein carboxylmethylation. Alternatively the radiolabeled immunoprecipitated proteins were detected by autoradiography.

Methylation of in Vitro Translated xENaC Subunits—For the studies examining the methylation of *in vitro* translated xENaC full-length *in vitro* translated α-, β-, or γ-xENaC were incubated with [^{3}H]AdoMet in the presence of 100 μM GTPγS and 50 μl of a carboxylmethyltransferase enzyme preparation for 1 h at room temperature. Base labile incorporation of methyl groups was assayed as described above.

Planar Lipid Bilayer Experiments—Planar lipid bilayers were painted with a fire polished glass rod over an aperture of 200-μm diameter in a polysterene chamber. The standard membrane forming solution consisted of a mixture of diphytanoyl-phosphatidylethanolamine/diphytanoyl-phosphatidylserine, 2:1 (w/w; final lipid concentration, 25 mg/ml in n-octane). A membrane capacitance of 250–300 picofarad was considered satisfactory for experimentation. The bilayer bathing solutions contained 100 mM NaCl buffered to pH 7.4 with 10 mM MOPS-Tris. The current-to-voltage converter was connected to the trans side of the bilayer chamber using Ag/AgCl electrodes and 3 M KCl 3% agar bridges. Thus, the trans compartment was held at virtual ground. Reconstituted proteoliposomes containing a mixture of α-, β-, or γ-rENaC proteins were spread over the trans side of a pre-formed bilayer held at -40 mV. This protocol provides a specific sidedness to incorporation of the channels, *i.e.* they will be oriented with the amiloride-sensitive (extracellular) side facing the trans solution and the cytoplasmic side facing the cis solution in over 90% of the incorporations.

Currents were monitored on a strip chart recorder and/or a digital storage oscilloscope and were stored unfiltered on a VCR tape. Current records were low pass filtered at 100 Hz through an 8-pole Bessel filter prior to acquisition using a Digidata 1200 interface and PCLAMP
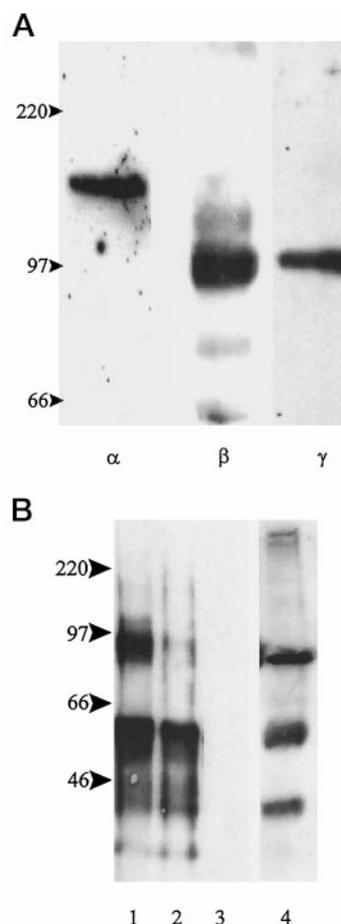
FIG. 1. **Specificity of the xENaC antibodies in A6 cells.** *A*, apical membranes from A6 cells were biotinylated as described under "Experimental Procedures," and biotinylated proteins were collected with streptavidin beads. Proteins were separated by SDS-PAGE and analyzed by Western blot with anti-α-, β-, or γ-xENaC antibody. *B*, A6 cell membranes were immunoprecipitated with either anti-β-xENaC (*lanes 1* and *2*) or γ-xENaC antibody (*lane 4*). *Lane 2* was done in the presence of the immunizing peptide. *Lane 3* was immunoprecipitated with preimmune serum. *Lanes 1–3* were probed with anti-β-xENaC. *Lane 4* was probed with anti-γ-xENaC antibody. This band was also competed away by preincubation with the immunizing peptide (not shown).

software. Single channel open probability (P_o) was calculated for at least 3 min of continuous recording using the equation: $P_o = I/(N \cdot i)$ where N is total number of channels, I is the mean current over the period of observation, and i is the unitary current. The total number of channels (N) present in bilayer in each experiment was determined by activating all of them with a hydrostatic pressure gradient. This procedure is derived from previous observations that the P_o for approaches 1 when a sufficient hydrostatic pressure gradient is imposed across the bilayer ENaC (11). Only membranes with single channels were used. DTT pretreatment of ENaCs was performed as described (12).

Statistics—Paired and non-paired t tests were performed using Sigma Stat Statistical Program (Jandel Scientific). Results were considered significant if $p < 0.05$.

RESULTS

Reactivity of Antibodies in A6 Cells—Because we wished to demonstrate channel subunits in the apical membrane, membrane proteins were isolated by selective apical surface biotinylation as described under "Experimental Procedures." Biotinylated apical membrane proteins were subjected to precipitation with Avidin beads, and reactive proteins were eluted into sample buffer and resolved by SDS-PAGE. After transferring to nitrocellulose, the proteins were analyzed by Western blot analysis using the anti-xENaC antibodies. As shown in Fig. 1*A*, the anti-β and -γ antisera recognize a 97-kDa apical membrane protein in A6 cells. By contrast the anti-α
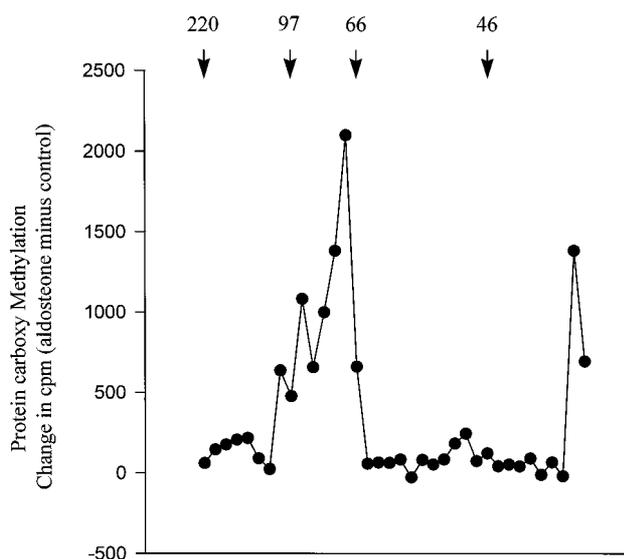
Methylation of β-xENaC Subunit Regulates Channel Activity

FIG. 2. **Aldosterone induced protein carboxylmethylation.** A6 cell membranes from cells treated with 10^{-6} M aldosterone for 3 h or diluent were incubated with [^{3}H]AdoMet and 100 μM GTPγS. Proteins were separated by SDS-PAGE, dried, sliced into 1–2-mm strips, and analyzed by the vapor phase method as described (19). The difference in base-labile counts between control and aldosterone treated cell membranes is shown ($n = 3$).
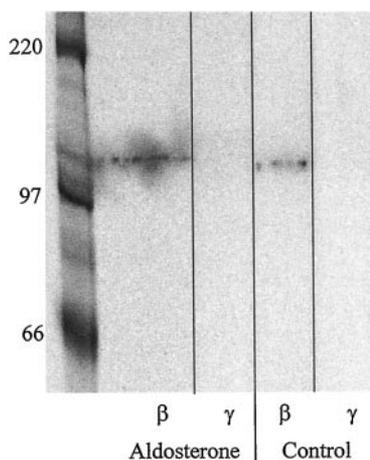

FIG. 3. **β-xENaC subunit is methylated in A6 cells.** Cell membranes from A6 cells treated with either aldosterone or diluent were carboxylmethylated *in vitro* as described under "Experimental Procedures" and then subjected to immunoprecipitation with either anti-β- or γ-xENaC. Samples were analyzed by SDS-PAGE and autoradiography ($n = 3$).

antibody recognized a 150-kDa protein. The predicted molecular mass of the glycosylated form of α xENaC is 75 kDa. This discrepancy may be due to the formation of a α-α dimer in native tissue, as has previously been suggested (11). The migration of α-ENaC at 150 and 180 kDa has also been observed by other groups (11).[2] All three proteins in A6 cells were competed away by pre-incubating the antisera with their respective peptide before Western blotting and were not detected when pre-immune serum was used (data not shown). Similar results were obtained with Western blot of proteins from whole cell lysates (data not shown).

Because these anti-xENaC subunit antibodies recognize the endogenous proteins, we next determined the ability of these antisera to immunoprecipitate xENaC from A6 cells. A6 lysates
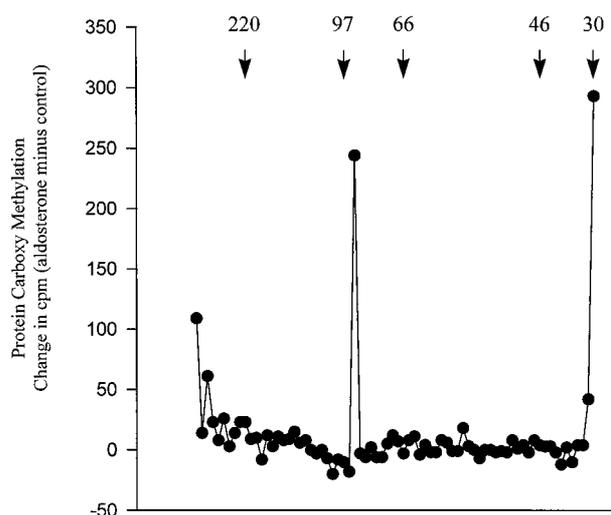
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FIG. 4. **Immunoprecipitation of the methylated β-xENaC subunit in response to aldosterone.** Cell membranes from A6 cells treated with either aldosterone or diluent were carboxylmethylated and subjected to immunoprecipitation with anti-β-xENaC antibody. Samples were analyzed by SDS-PAGE, dried, cut into 1–2 mm strips, and analyzed for base labile counts. Only a spike at 97 kDa was seen ($n = 4$). A representative gel slice is shown.

were immunoprecipitated with the anti-β or -γ subunit antibody, and reactive proteins were eluted into sample buffer and resolved by SDS-PAGE. After transferring to nitrocellulose, the samples were probed with either the anti-β or -γ antibody. As shown in Fig. 1*B* the anti-β (*lane 1*) or -γ (*lane 4*) antibody, both immunoprecipitate a 97-kDa band that was detected by Western blotting using their respective antisera. The 97-kDa band was not seen when either pre-immune serum (*lane 3*) or antibody preincubated with immunizing peptide (*lane 2*) was used in the reaction. These results demonstrate that the mature forms of the β and γ subunits isolated from A6 migrate as 97 kDa.

Methylation of A6 Membranes and Reactivity with xENaC Subunit Antibodies—After successfully generating and characterizing the specific antisera against the three xENaC subunits, we tested the hypothesis that one or more of these subunits act as substrates for carboxylmethylation. Sariban-Sohraby (4) has demonstrated that a 95-kDa apical membrane protein was methylated in response to aldosterone, and this process was blocked by spironolactone. In addition, this study showed that a similar protein may be isotopically labeled *in vitro* in membranes from A6 cells when incubated with [^{3}H]AdoMet in the presence of GTPγS. This apparent methylation was increased in membranes from cells pretreated with aldosterone.
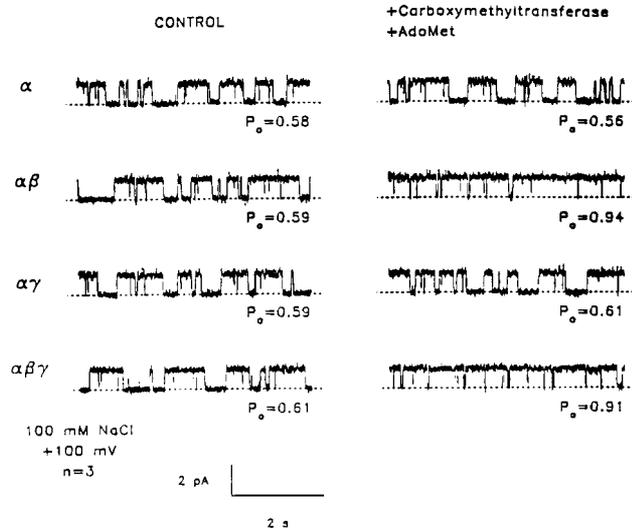
To determine whether these isotopically labeled proteins represent carboxylmethyl esters, the vapor phase method of Clarke (19) was employed. As shown in Fig. 2, a large number of proteins are carboxylmethylated, including a band at 97 kDa. To determine whether this methylated 97-kDa protein represents a Na$^+$ channel subunit, A6 cells were methylated using [^{3}H]AdoMet, and methylated cell membranes were subjected to immunoprecipitation with specific anti-xENaC antibodies. As shown in Fig. 3, the anti-β antibody immunoprecipitated a 97-kDa protein from both aldosterone-treated cells and from control cells incubated with GTPγS. In the absence of GTPγS, the anti-β antibody did not immunoprecipitate any methylated protein (data not shown). By contrast, the anti-γ antibody did not immunoprecipitate a methylated protein under any condition.

Alternatively, methylated cell lysates subjected to immunoprecipitation with the anti-β- or γ-xENaC antibody were re-
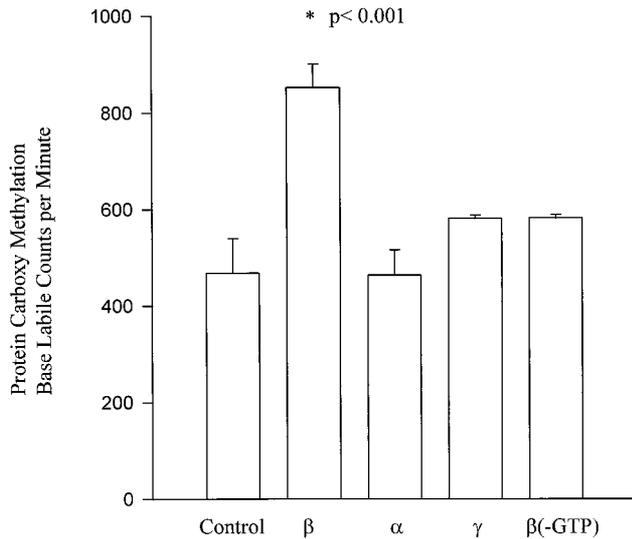
FIG. 5. **Methylation of the *in vitro* translation of the β- and γ-xENaC subunits.** Full-length cDNA for α-, β-, and γ-xENaC was translated *in vitro* using a reticulocyte lysate system. Translated proteins were carboxylmethylated with methyltransferase prepared from A6 cell membranes from cells grown on plastic Petri dishes and analyzed for base-labile incorporation of [3H]methyl groups. Incorporation of isotope above background was seen only for β-xENaC incubated in the presence of GTP. $n = 4$ for each subunit.

solved by SDS-PAGE. The gel was sliced, and the gel slices were exposed to NaOH as described under "Experimental Procedures" to allow determination of base labile counts. As shown in Fig. 4, the protein that was immunoprecipitated with the anti-β-xENaC antibody has been carboxylmethylated. To confirm that β-xENaC is a substrate for methylation, full-length, *in vitro* translated xENaC subunits were incubated with [3H]AdoMet in the presence or absence of GTPγS. A carboxylmethyltransferase enzyme preparation, prepared as described under "Experimental Procedures," was included in the reaction mixture. This enzyme preparation did not contain detectable amounts of any xENaC subunits when examined by Western blot (not shown). The results show that the *in vitro* translated β-xENaC protein was a suitable substrate for methylation in the presence of but not in the absence of GTPγS (Fig. 5). We were unable to demonstrate methylation of either *in vitro* translated α- or γ-xENaC. Taken together these results indicate that in A6 cells, only the β-xENaC subunit is carboxylmethylated in the response to aldosterone.

Methylation and Channel Activity—To determine whether methylation of ENaC subunits reconstituted in lipid bilayers altered channel activity, various combinations of rENaC subunits were incorporated into lipid bilayers and subjected to enzymatic methylation. Channel activity is shown prior (Fig. 6,
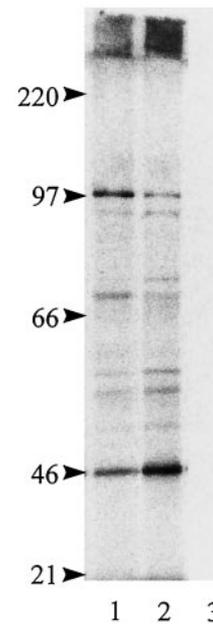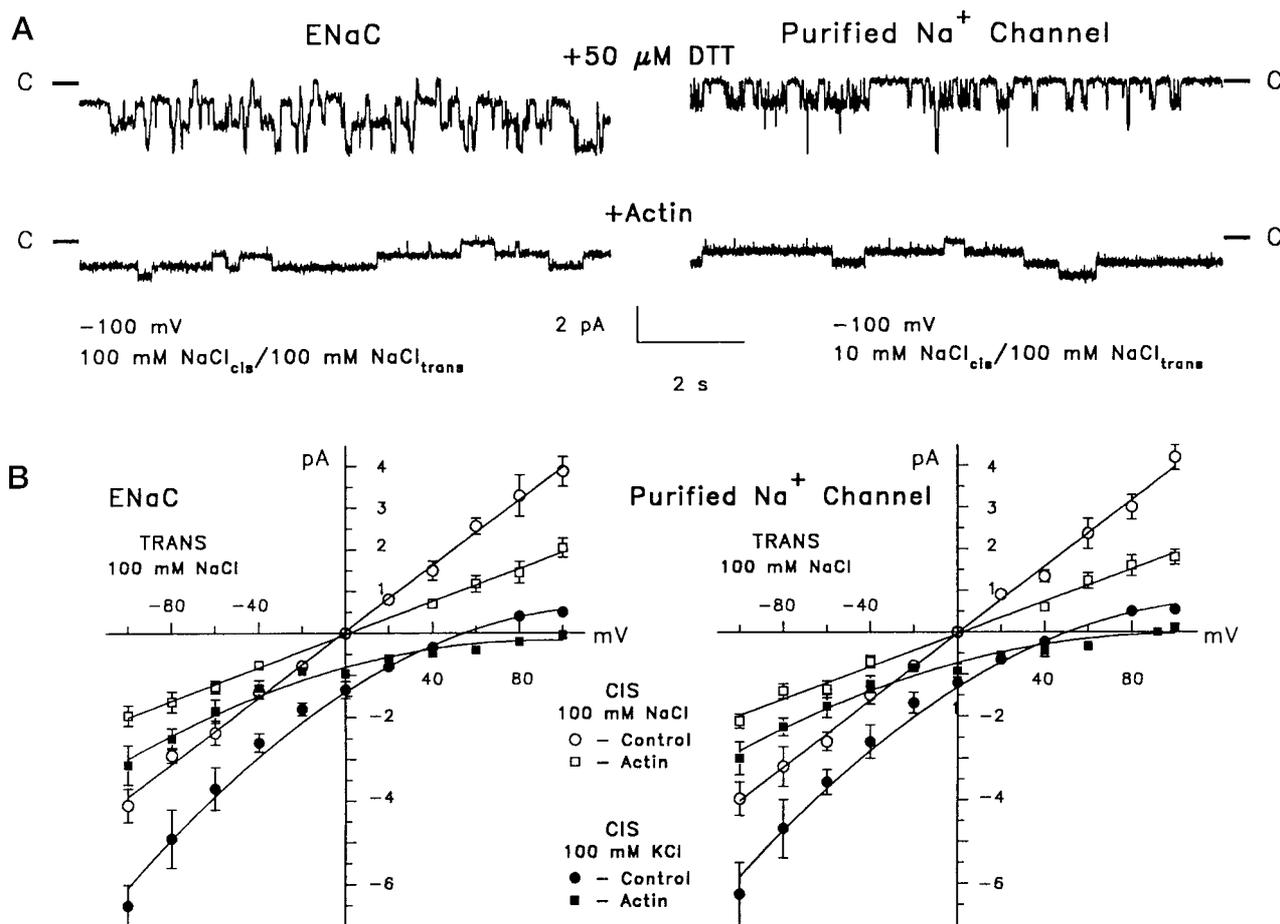

FIG. 6. **The effect of carboxylmethylation on various combinations DTT-pretreated rENaCs reconstituted in lipid bilayers.** Bilayers were bathed with symmetrical 100 mM NaCl, 10 mM MOPS-Tris buffer (pH 7.4). Traces were recorded at the holding potential of +100 mV and are representative of at least three separate experiments performed with each composition of the channel. Carboxylmethyltransferase was used at a final concentration of 7 μg/ml. 10 mM AdoMet and 100 μM GTPγS were also added to cis compartment. Shown is a representative tracing ($n = 3$).

FIG. 8. **Immunoprecipitation using the anti-β-xENaC antibody recognizes the same proteins immunoprecipitated with the immunopurified Na+ channel complex.** A6 cells were labeled for 3 h with Easy Tag Express [35S] and subjected to immunoprecipitation with the immunopurified complex (*lane 1*) or the anti-β-xENaC antibody (*lane 2*). Samples were separated by SDS-PAGE and analyzed by autoradiography. An identical pattern of proteins with molecular masses 220, 97, 66, 45, and 30 kDa is seen. *Lane 3* is a negative control. Cells were treated identically and immunoprecipitated without primary antibody ($n = 2$).

FIG. 7. **The immunopurified Na+ channel complex contains the α-, β-, and γ-xENaC subunits.** A6 cell membranes were immunoprecipitated with α-, β-, or γ-xENaC antibody, and paired samples were immunoprecipitated with antibody to the immunopurified Na+ channel complex. Proteins were separated by SDS-PAGE and probed with anti-α-, β-, or γ-xENaC antibodies. Shown is a representative autoradiogram ($n = 2$).

FIG. 9. **Single channel records of αβγ-rENaC and immunopurified renal amiloride-sensitive sodium channel reconstituted into planar lipid bilayers.** *A*, bilayers containing ENaC were bathed with symmetrical solutions of NaCl (100 mM MOPS-Tris, pH 7.4. Bilayers with immunopurified bovine renal Na$^+$ channels (23) were bathed with asymmetrical salt solutions of NaCl (cis compartment contained 10 mM NaCl, 10 mM MOPS-Tris, pH 7.4; *trans* compartment contained 100 mM NaCl, 10 mM MOPS-Tris, pH 7.4). Traces shown are typical of at least four independent experiments. Holding potential was −100 mV referreed to the virtually grounded *trans* chamber. Current records were filtered at 300 Hz through an 8-pole Bessel filter and acquired at 1 kHz sampling rate using a Digidata 1200 interface and pCLAMP software. For illustration purposes, records were filtered at 60 Hz using pCLAMP software. *B*, single channel current voltage relationship of ENaC and immunopurified bovine renal amiloride-sensitive sodium channel reconstituted into planar lipid bilayers under symmetrical (100 mM NaCl$_{cis}$/100 mM NaCl$_{trans}$, 10 mM MOPS-Tris, pH 7.4, *open symbols*) and bi-ionic (100 mM KCl$_{cis}$/100 mM KCl$_{trans}$, 10 mM MOPS-Tris, pH 7.4, *closed symbols*) conditions. *Data points* and *error bars* represent the means ± S.D. of at least four separate experiments.

left panel) and after (*right panel*) the addition of AdoMet, GTPγS, and carboxylmethyltransferase to the side opposite to which amiloride inhibited channel activity, *i.e.* the putative cytoplasmic side. As shown in Fig. 6, α-, β-, and γ-rENaC activity was increased in response to methylation (*bottom panel*) as was the α, β-rENaC combination (*second panel* from the top). By contrast, when α-rENaC was incorporated alone (*top panel*) or in combination with γ-rENaC (*third panel* from the top), there was no increase in channel activity in response to methylation. This increase in ENaC activity was only evident when the carboxylmethylation reaction mixture was added to the presumptive cytoplasmic side. These results demonstrate that methylation of the β-ENaC subunit results in an increase of open probability of the channel complex.

The Immunopurified Na$^+$ Channel Complex Proteins Contains All Three Individual Subunits—Because our earlier studies had demonstrated that methylation of a subunit of the immunopurified Na$^+$ channel complex of approximately the same size as β-xENaC resulted in similar increases in channel activity when reconstituted in bilayers (5), we next sought to determine the relationship between these proteins. Immunoprecipitations of channel proteins from A6 cells were preformed using a polyclonal antibody to the immunopurified complex and the antibodies to all three xENaC subunits. All four immuno-

precipitations were analyzed with the anti-xENaC antibodies. Because our α-xENaC antibody had not proved useful for immunoprecipitation (data not shown), we employed an antibody to α-xENaC developed in collaboration with Smith, Kleyman, and colleagues.[2] This antibody was raised against a peptide corresponding to amino acids 107–125 of α-xENaC. The antibody specifically immunoprecipitates *in vitro* translated α-xENaC but not β- or γ-xENaC. Immunoprecipitation of α-xENaC was blocked by excess competing peptide. The anti-α-xENaC antibody specifically recognizes polypeptides of ∼150 and ∼180 kDa on Western blots of A6 apical membranes, and these bands are competed away by preincubation with excess immunizing peptide.[2] The results shown in Fig. 7 demonstrate that all three xENaC subunits were recognized by their respective antibodies in the immunoprecipitated channel complex. We then compared immunoprecipitation of A6 proteins with the anti-β antibody and the polyclonal antibody to the immunoprecipitated channel complex in cells metabolically labeled with Easy Tag Express [35S]. As shown in Fig. 8, a complex of proteins similar in size to those comprising the immunopurified channel complex (8) was visualized with each antibody.

These results suggest a rationale for the concordance between our current and previous results (5) with methylation of the two channel complexes; namely, they contain the same

active channel constituents. One problem with this interpretation is that the kinetics of ENaC when reconstituted in bilayers are different from those observed for ENaC when that complex is expressed either in *Xenopus* oocytes or native tissues (8). We have previously suggested that this may be due to the conditions of reconstitution (8) and therefore sought conditions of reconstitution where the kinetics of the reconstituted channel complex would resemble those described for ENaC. When ENaC-containing proteoliposomes are pre-treated with 50 μM DTT, the concerted gating behavior of the channel is disrupted and only a single 13 picosiemens conductance element can be incorporated into the bilayer (see Fig. 6).[3] Subsequent to the addition of actin, single channel conductance was reduced to 6 picosiemens, and the open and closed kinetics of the channel slowed to the second time range.[3] A direct comparison between $\alpha\beta\gamma$-rENaC and immunopurified renal Na$^+$ channel behavior under these conditions is shown in Fig. 9. Moreover, determination of Na$^+$ *versus* K$^+$ permeability ratios (P_{Na}^+/P_K^+) from reversal potentials measured under bi-ionic salt conditions revealed that actin increased P_{Na}^+/P_K^+ from 8:1 to 54:1 for ENaC and from 7:1 to 40:1 for the purified Na$^+$ channel. Addition of AdoMet, GTPγS, and carboxylmethyltransferase to the cis (or "cytoplasmic") bathing medium activated only β-containing ENaC channels. Similar results were obtained in non-DTT-pretreated channels (data not shown).

DISCUSSION

During the early phase of its action, aldosterone increases Na$^+$ reabsorption across responsive epithelia by an increase in the activity of the epithelial Na$^+$ channel, ENaC (8, 20, 21). During the first 4 h following hormone addition, this occurs without synthesis or addition of new channel subunits to the apical membrane (8, 20, 21). One hypothesis that has been proposed for the mechanism of this early channel activation involves carboxylmethylation of an apical membrane protein in response to aldosterone (1–4). We have previously demonstrated that carboxylmethylation of a 95-kDa subunit of the immunopurified channel results in activation of that channel complex when reconstituted in planar lipid bilayers (5). The current studies were designed to determine whether similar results could be observed with carboxylmethylation of ENaC subunits. To carry out these studies, it was necessary to generate antibodies of ENaC subunits in our cellular model system, A6. We also sought to determine whether xENaC subunits could be carboxylmethylated *in vitro* and what effect this might have on kinetics of the channel reconstituted in lipid bilayers.

The results of the current study demonstrate that the β-xENaC is methylated, whereas α- and γ-xENaC are not. The carboxylmethylation is stimulated by GTPγS, as previously observed (4, 5) and is enhanced in membranes from cells pre-treated with aldosterone when compared with controls (5). Methylation of ENaC reconstituted in planar lipid bilayers results in increased channel activity similar to the result obtained previously with the immunopurified channel complex (4), *i.e.* only when the β-ENaC subunit is present.

These results led us to examine the relationship between the

immunopurified complex and xENaC. The antibodies that recognize the three subunits of xENaC also recognize similarly sized proteins within the immunopurified complex, and this complex of proteins may be immunoprecipitated using our antibody to β-xENaC. Both channels, when reconstituted in lipid bilayers, display activation when the 97-kDa subunit is carboxylmethylated. This evidence strongly suggests that the two preparations contain identical channel proteins.

The site of the relevant methylation on β-xENaC has not been examined in this paper but is the subject of ongoing research. Because the enzyme is active from the putative cytoplasmic side in bilayer reconstitution studies, we speculate that the site may likely be located on either the NH$_2$-terminal or COOH-terminal cytoplasmic domains. It is interesting to hypothesize that mutations in the region of this protein responsible for this form of regulation would result in unregulated channel activity. Inspection of the primary structure of the β-xENaC does not reveal any obvious or classical sites for regulatory carboxylmethylation, so this reaction may represent a site-specific modification similar to those described for prokaryotes (22).

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