Insulin-induced phosphorylation of ENaC correlates with increased sodium channel function in A6 cells

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Submitted 15 January 2004; accepted in final form 30 August 2004

Zhang, Yu-Hua, Diego Alvarez de la Rosa, Cecilia M. Canessa, and John P. Hayslett. Insulin-induced phosphorylation of ENaC correlates with increased sodium channel function in A6 cells. Am J Physiol Cell Physiol 288: C141–C147, 2005. First published September 8; doi:10.1152/ajpcell.00343.2004.—The purpose of this study was to determine whether there is a correlation between phosphorylation and activity of the epithelial sodium channel (ENaC). The three subunits that form the channel were immunoprecipitated from A6 cells by using specific polyclonal antibodies after labeling cells with 35S or 32P. When immune complexes were resolved on SDS-PAGE, the α-subunit migrated at 85 and 65 kDa, the β-subunit at 115 and 100 kDa, and the γ-subunit at 90 kDa. Exposure to aldosterone or insulin markedly increased phosphorylation of the α-subunit, in resting cells. Furthermore, the administration of aldosterone or insulin markedly increased phosphorylation of the β- and γ-subunits. Furthermore, the administration of aldosterone or insulin markedly increased phosphorylation of the β- and γ-subunits by the transfer of phosphate to residues located in the carboxy terminus (14). If these observations were confirmed in cells with endogenous ENaC and genetic regulation of signals that control sodium channel activity, then these findings would imply not only that phosphorylation of ENaC subunits is an important factor controlling the activity of the sodium channel but also that both aldosterone and insulin share a common regulatory mechanism to stimulate sodium transport.

In this study we used A6 cells that express endogenous ENaC and the machinery to stimulate electrogenic sodium transport when exposed to aldosterone and other agonists that act at the level of the cell membrane, such as insulin and vasopressin. We examined whether the subunits of ENaC were phosphorylated in the resting state and after exposure to aldosterone or insulin. In addition, we examined the phosphorylation of ENaC subunits in A6 cells pretreated with aldosterone and stimulated with insulin, because numerous studies (5, 10, 12, 13) have demonstrated a synergistic increase in sodium transport under this experimental condition. Our results demonstrate phosphorylation of all three endogenously expressed ENaC subunits in intact cells. Furthermore, this study links for the first time an increase in subunit phosphorylation with changes in sodium transport in cells expressing endogenous ENaC.

MATERIALS AND METHODS

Cell culture. Experiments were performed on A6S2 cells derived from the kidney of Xenopus laevis as previously reported (13). Cells were grown to confluency on Falcon permeable supports with a diameter of 25 mm and were maintained in modified DMEM (GIBCO, Grand Island, NY) supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), 10% fetal bovine serum (FBS), and NaHCO3 (8 mM), pH 7.6. FBS was removed after seeding, when transmembrane resistance was maximal. Cells were grown to confluency on Falcon permeable supports with a diameter of 25 mm and were maintained in modified DMEM (GIBCO, Grand Island, NY) supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), 10% fetal bovine serum (FBS), and NaHCO3 (8 mM), pH 7.6. FBS was removed after seeding, when transmembrane resistance was maximal. Cells were maintained at 27°C in a humidified incubator gassed with 1% CO2. For sodium transport studies, cells were seeded on Millipore-HA culture inserts (Bedford, MA) that were 12 mm in diameter, and transport was expressed as the equivalent short-circuit current (Ieq), as described previously (13).

Plasmid constructs. Full length α and β Xenopus ENaC subunit cDNAs (11) were amplified by PCR and subcloned in pcDNA 3.1 (Invitrogen, Carlsbad, CA). The forward primer for PCR contained a

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consensus Kozak sequence, and the reverse primer contained the coding sequence of a FLAG epitope before the stop codon. Construct identity was checked by sequencing at the HHMI/Keck facility at Yale University.

**Antibody generation and Western blot analysis.** Polyclonal antibodies were raised in rabbit against α-, β-, and γ-subunits by injection of glutathione S-transferase (GST) fusion proteins containing the sequences H544–N632 for α, T149–N226 for β, and R566–L660 for γ. Their specificity has been previously reported (1). In addition, polyclonal antibodies were raised in rabbit against amino acid sequences in the carboxy termini of the α-subunit (P561–D581) and the β-subunit (P5890–G609) and conjugated to bovine serum albumin for injection. These peptides were synthesized at the HHMI/Keck facility at Yale University. Antibody specificity was studied by performing Western blot analysis. Briefly, constructs expressing the α- or β-subunits of ENaC were transiently transfected into human embryonic kidney (HEK) cells with Lipofectamine 2000 (Invitrogen) for 3–4 h. The half-life of ENaC was free of methionine and cysteine and were then incubated in the same medium for 20 min. Cells were labeled with a mixture of [35S]methionine and [35S]cysteine (150 μCi/ml; Amersham Pharmacia Biotech, Piscataway, NJ) for 3–4 h. The half-life of ENaC subunits in A6 cells is 40–60 min (1). Therefore, this protocol labels the three subunits to steady-state levels. After the labeling period, cells were washed with ice-cold phosphate-buffered saline, scraped with a rubber policeman, recovered by brief centrifugation in an Eppendorf microfuge, and lysed in 50 μl of lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 2 mM PMSF, and 5 μg/ml each of pepstatin, leupeptin, and aprotinin). After 15 min, lysates were cleared by centrifugation at 10,000 rpm for 10 min at 4°C and SDS was added to the supernatant to a concentration of 4.7%. Samples were then heated at 95°C for 5 min. After denaturation, samples were diluted in 1.4 ml of lysis buffer and the antibody of interest was added. Samples were then incubated for 4 h at room temperature. After incubation, 50 μl of a 50% slurry of protein A-Sepharose CL-4B beads (Sigma Aldrich) were added to each sample and tubes were placed on a rocker for 1 h at room temperature. The beads were then washed three times with lysis buffer and then three times with a solution containing 10 mM Tris·HCl, pH 7.4, 2 mM EDTA, and 0.1% SDS. Protein was eluted from the beads in 70 μl of Laemmli sample buffer. As previously described by investigators from this laboratory (1), the amounts of antisera and protein A beads added to recover the immune complexes from the lysates were titrated to ensure complete recovery of antigens. The batches of antibody used in all experiments were the same, and the protein A beads were of equal binding capacity. The total amount of protein in the lysate was kept constant. The immune complexes were resolved in SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Membranes were dried and exposed to Biomax MR film (Eastman Kodak, Rochester, NY) with a Biomax LE intensifying screen at ~80°C. Densitometric analysis was performed in a GS-800 calibrated densitometer using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

In studies performed with [32P]orthophosphate, the cells were initially washed in phosphate-free DMEM three times and then maintained in phosphate-free solutions until lysis was performed. The isotope (1 μCi/ml) was added to the basal side of the membrane, and cells were incubated for 3 h (14). The procedures for immunoprecipitation performed otherwise were the same as described above, except that phosphatase inhibitors (0.2 mM sodium orthovanadate, 50 mM sodium fluoride, and 30 mM sodium pyrophosphate) were added to the lysis buffer. Experimental quantification of changes in subunit phosphorylation included between three and six replicate samples for each condition.

**Statistical analysis.** Data points represent the average of n independent experiments (±SE). Differences between groups were evaluated with the nonpaired t-test. The P and n values are given in the text of figure legends when appropriate.

**RESULTS**

**Characterization of specific antibodies against Xenopus ENaC subunits.** The rabbit polyclonal antibodies used in these studies were generated against fusion proteins containing sequences of Xenopus ENaC (xENaC) α-, β-, and γ-subunits. In a previous report (1), investigators from our laboratory confirmed the specificity of the antibodies according to their ability to immunoprecipitate the three subunits from transiently transfected A6 cells. Further analysis of A6 cells grown on filters showed similar results when A6 lysates were subjected to immunoprecipitation or examined using Western blot analysis (1). The bands were absent in preimmune serum or when the antibody binding was competed with excess fusion protein. Both methods of analysis showed that the α-subunit was represented by a band of 85 kDa and by a second band that migrated at 65 kDa and represents a posttranslational modification of the 85-kDa form (1). The β-subunit had bands at 100 and 115 kDa due to distinct glycosylation patterns, whereas the γ-subunit migrated at 90 kDa (1).

To provide further verification that the bands detected with anti-α- and anti-β-subunit antisera are specific, we raised a new set of rabbit polyclonal antibodies against short amino acid sequences corresponding to the carboxy termini of xENaC α- and β-subunits. Figure 1A shows a Western blot analysis of protein lysates from HEK cells transfected with expression vectors containing the full-length cDNAs of α- and β-subunits. Identical bands were observed when the vectors were probed with anti-FLAG or with anti-ENaC antibodies, indicating that each antisera raised against α- and β-subunits recognized the appropriate subunit of xENaC. The lower band of the α-subunit was not exhibited in the transfected HEK cells. Figure 1B shows an autoradiograph of xENaC α- and β-subunits immunoprecipitated from lysate of [35S]-labeled A6 cells and resolved on SDS-PAGE. α- and β-subunits were immunoprecipitated with either anti-fusion protein antibodies (lane 1) or anti-peptide antibodies (lane 2). The bands shown in Fig. 1B were identical when the two types of antibodies were compared. A similar comparison for the γ-subunit could not be made because the anti-peptide antibody had a low potential for immunoprecipitation. In contrast to transfected HEK cells, the 65-kDa band of the α-subunit was present in A6 cell lysates and was detected by both types of antibodies. These results further confirm the specificity of the two bands of the α-subunit migrating at 85 and 65 kDa and the two bands of the β-subunit migrating at 100 and 115 kDa. The anti-fusion protein antibodies against the three subunits (1) were used for the rest of the experiments in this study.

**Effects of insulin and aldosterone on biosynthesis of subunits.** We next examined the level of expression of xENaC subunits in A6 cells to determine whether aldosterone or insulin increases the abundance of the channel subunits. Cells grown on permeable membranes were exposed to 1 μM aldo-
sterone for 16 h and were labeled with $^{35}$S during the last 3–4 h. Cells were analyzed without further treatment or after addition of 100 nM insulin during the final 30 min. All three xENaC subunits were immunoprecipitated from the lysates and resolved on SDS-PAGE. Figure 2 shows that aldosterone induced a robust increase in protein abundance in all three subunits (varying from a 5- to 8-fold increase in 3 independent experiments), which confirms and extends recent observations (1) that aldosterone caused a progressive increase in subunit abundance over a period of 6 h. The addition of insulin, however, did not significantly change protein abundance when used alone or when added to A6 cells pretreated with aldosterone.

**Phosphorylation of xENaC subunits.** Using Madin-Darby canine kidney (MDCK) cells cotransfected with rat $\alpha$, $\beta$, and $\gamma$ ENaC subunits, Shimkets et al. (14) reported that the $\beta$- and $\gamma$-subunits, but not the $\alpha$-subunit, were phosphorylated on the carboxy termini under basal conditions. In addition, they observed an increase in phosphorylation of the $\beta$- and $\gamma$-subunits, but not the $\alpha$-subunit, after exposure to aldosterone or insulin (14). The following studies were performed to determine whether xENaC subunits are phosphorylated in cells that express endogenous ENaC. A6 cells were exposed to aldosterone for 16 h to maximize xENaC subunit expression and then metabolically labeled with $^{35}$S or $^{32}$P. The three xENaC subunits were recovered by immunoprecipitation and resolved by SDS-PAGE. Figure 3 shows the comparison of bands representing subunit protein, detected on SDS-PAGE by $^{35}$S labeling (lane 1), and phosphorylated bands, detected by $^{32}$P labeling (lane 2). It is apparent that phosphorylation of the $\alpha$-subunit is restricted to the lower migrating band at 65 kDa, whereas both bands of the $\beta$-subunit and the single band of the $\gamma$-subunit were phosphorylated.

**Phosphorylation of xENaC subunits before and after stimulation by aldosterone or insulin.** Because previous studies in transfected cells showed that ENaC $\beta$- and $\gamma$-subunits were phosphorylated in the basal state as well as after stimulation by various sodium transport agonists (14), we wanted to examine whether ENaC subunit phosphorylation exhibited similar characteristics in cells that endogenously express channels and the intracellular machinery for the regulation of electrogenic sodium transport.
We first studied the response of electrogenic sodium transport to aldosterone and insulin stimulation in A6 cells. Incubation of filters with 1 μM aldosterone overnight increased transepithelial $I_{eq}$ from $6.2 \pm 0.7$ to $25.3 \pm 1.0$ μA/cm² (Fig. 4A), whereas 30-min stimulation with 100 nM insulin increased it from $6.4 \pm 0.5$ to $16.6 \pm 1.1$ μA/cm² ($\Delta I_{eq} = 10.2$ μA/cm²). These values are in good accord with previously published measurements (1, 5, 7, 10, 12, 13). When insulin was applied to cells pretreated with aldosterone, a synergistic effect was observed. $I_{eq}$ increased from $26.8 \pm 1.1$ to $52.9 \pm 2.1$ μA/cm² ($\Delta I_{eq} = 26.1$ μA/cm²).

Next, we studied $\alpha$ENaC subunit phosphorylation under the same conditions used for transport studies. After metabolic labeling with $^{32}$P and agonist treatment, cells were lysed and

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Fig. 4. Phosphorylation of ENaC subunits before and after stimulation with aldosterone and insulin. A: equivalent short-circuit current ($I_{eq}$) values for A6 cells grown on filters before (control) and after treatments with 1 μM aldosterone (Aldo) for 19.5 h and 100 nM insulin for 30 min. Each bar represents the average $I_{eq}$ (±SE) from 6 independent filters. B: control cells or cells pretreated with 1 μM aldosterone overnight were labeled with $^{32}$P for 3 h. When indicated, 100 nM insulin was added for the last 30 min of labeling. $\alpha$ENaC $\alpha$-, $\beta$-, and $\gamma$-subunits were then immunoprecipitated from cell lysates with anti-fusion protein antibodies and resolved on SDS-PAGE. Representative autoradiographs are shown. Arrows indicate migration of molecular mass markers (kDa). C: bars represent the average values (±SE) of phosphate incorporation into each subunit, normalized to the control group ($n = 3–5$). *$P < 0.05$ compared with control. **$P < 0.05$ compared with Aldo.
ENaC regulation by phosphorylation

\(\alpha\)-ENaC subunits were recovered by immunoprecipitation. Figure 4B shows representative autoradiographs of immunoprecipitation products resolved in SDS-PAGE. All three subunits were phosphorylated in the basal state. The \(\alpha\)-subunit is phosphorylated only at the 65-kDa band in the resting state as well as after aldosterone stimulation. Quantification of the autoradiographs demonstrates that aldosterone induced a five- to eightfold increase in \(32^P\) levels for all three subunits (Fig. 4C, \(n = 3-5, P < 0.05\)). The increase in \(32^P\) levels was proportional to the aldosterone-induced increase of protein abundance (Fig. 2), suggesting that phosphorylation was dependent on the amount of protein available. It should be noted, however, that the large increase in subunit abundance induced by aldosterone could mask smaller increases in the fraction of phosphorylated subunits or in the relative content of phosphate per subunit.

Insulin stimulation increased phosphorylation of the \(\alpha\), \(\beta\), and \(\gamma\)-subunits 1.7- to 2.0-fold above baseline (Fig. 4C). This effect was not dependent on the abundance of protein because the effect was not dependent on the abundance of protein because and \(H9253\). This result correlates well with the synergistic increase in sodium transport induced by insulin when used in aldosterone-pretreated cells (Fig. 4A).

Effect of protein kinase C inhibitor chelerythrine on phosphorylation of ENaC subunits. Previous results from our laboratory (13) suggest that the increased sodium transport induced by insulin is dependent on activation of protein kinase C (PKC). Therefore, we examined whether chelerythrine, a highly specific and potent (IC\(_{50}\)) inhibitor of PKC, has any effect on the increase in ENaC subunit phosphorylation induced by insulin. We first studied the effect of chelerythrine on electrogenic sodium transport and insulin stimulation. Incubation of filters with 100 nM insulin for 30 min increased \(I_{eq}\) from 2.5 \pm 0.4 to 6.3 \pm 0.9 \mu A/cm\(^2\) (Fig. 5A). Cells preincubated for 15 min with different concentrations of chelerythrine showed a dose-dependent decrease in the response to insulin. The decrease was already apparent with 3 \mu M chelerythrine and was complete with 10 \mu M chelerythrine (Fig. 5A). With this concentration \(I_{eq}\) decreased below basal values (1.2 \pm 0.4 \mu A/cm\(^2\)). In a separate experiment, we tested whether chelerythrine had toxic effects on the cells. When insulin was applied to cells pretreated for 15 or 30 min with 10 \mu M chelerythrine, the \(I_{eq}\) value was 4.2 \pm 0.5 or 1.8 \pm 0.5 \mu A/cm\(^2\), respectively, both below basal transport values. An increase in the dose of chelerythrine to 20 \mu M made the effect even more pronounced, bringing \(I_{eq}\) close to zero with 15 min pretreatment and to zero with 30-min pretreatment. After treatments, the medium was changed and the cells were returned to the incubator overnight. After the recovery period, the cells that had been treated with 10 \mu M chelerythrine showed \(I_{eq}\) values indistinguishable from control values and were still responsive to insulin stimulation. In contrast, when cells were pretreated with 20 \mu M chelerythrine, \(I_{eq}\) did not recover and was unaffected by insulin stimulation, showing that this concentration had toxic effects on A6 cells (data not shown).

We then studied chelerythrine effects on \(\alpha\)ENaC subunit phosphorylation (Fig. 5, B and C). On the basis of transport studies, we chose to treat the cells with 10 \mu M chelerythrine for 30 min, because this condition gives maximal effects on \(I_{eq}\) without toxic effects. Cells were metabolically labeled with \({}^{32}P\), and phosphorylation of ENaC \(\alpha\)-subunit was determined by immunoprecipitation after pretreatment with chelerythrine and/or stimulation with 100 nM insulin for 30 min. Insulin increased \(\alpha\)ENaC \(\alpha\)-subunit phosphorylation 1.6-fold (Fig. 5C), whereas preincubation with 10 \mu M chelerythrine for 30 min abrogated the increase in phosphorylation of the \(\alpha\)-subunit induced by insulin and decreased it to 40% below the basal value in control cells (Fig. 5C, \(n = 6, P < 0.01\)). This experiment was repeated twice with equal results. A similar effect was detected on the phosphorylation levels of \(\beta\)- and \(\gamma\)-subunits (data not shown).

Discussion

The ENaC serves to regulate sodium balance for the organism by controlling the fractional excretion of filtered sodium in the distal nephron. Since ENaC was cloned a decade ago (2, 3),
significant progress has been made in understanding its regulation at the molecular level, but there remain many questions about the signaling mechanisms that link the activated receptors to the increase in sodium channel activity. The present study was performed to determine whether there is a correlation between the activity of the sodium channel and the phosphorylation state of one or more of the three subunits that form the channel.

Shimkets et al. (14) examined the phosphorylation of ENaC in MDCK cells stably transfected with the three subunits that form the channel. It was found that β- and γ-subunits, but not α-subunits, were phosphorylated in the basal state in serum and threonine residues located in the carboxy terminus. In contrast, our experiments demonstrate phosphorylation in all three subunits of endogenously expressed ENaC in A6 cells. An unexpected finding was the phosphorylation of the 65-kDa band in the complete absence of phosphorylation of the 85-kDa band of the α-subunit. The lack of phosphorylation of the α-subunit when transfected into MDCK cells (14) may be explained by the lack of machinery to produce the appropriate posttranslational modification that gives rise to the 65-kDa form of the α-subunit (1). Alternatively, the 65-kDa form could be specific of A6 cells. The modification is not likely the product of cleavage from the 85-kDa band protein because both the amino and carboxy termini were present in the two bands detected when a green fluorescent protein α-subunit fusion protein was transfected in A6 cells (1). The 65-kDa protein appears to contain disulfide bridges resistant to reducing agents (1). The appearance of the 65-kDa protein is accompanied by the acquisition of resistance to endoglycosidase H digestion (1), indicating the presence of complex glycosylation in this form of the α-subunit. This implies that the phosphorylation process that affects the 65-kDa form has to occur in Golgi or post-Golgi compartments. Also, the fact that cell-surface biotinylation experiments show both the 85- and 65-kDa forms in the plasma membrane (1) indicates that phosphorylation of the α-subunit is not a requirement for traffic of the protein to the cell surface. On the other hand, both bands of the β-subunit and the single band of the γ-subunit were phosphorylated in A6 cells. The upper band of the β-subunit has complex glycosylation, whereas the lower band of the β-subunit and the γ-subunit are core glycosylated (1). Therefore, it is not possible to establish a relationship between the maturation of the β- and γ-subunits and their subcellular localization, making it impossible to distinguish whether the phosphorylation takes place only at the plasma membrane or also at some intracellular compartments.

Chigaev et al. (4) approached the same question with an in vitro assay in which GST fusion proteins containing the cytoplasmic domains of the ENaC subunits were used as phosphorylation substrates. They showed that the three ENaC subunits intracellular tails were phosphorylated by selective fractions of cytoplasm derived from rat colon. As in the study by Shimkets et al. (14), 32P was incorporated into threonine and serine residues, with no evidence of phosphorylation of tyrosines.

Shimkets et al. (14) also demonstrated that phosphorylation of the β- and γ-subunits was increased when cells were exposed to aldosterone or insulin. Therefore, they suggested that phosphorylation of ENaC might, in fact, control its functionality. In the present study, semiquantitative analysis of ENaC subunit phosphorylation in A6 cells (Fig. 4) demonstrated that insulin stimulation increases phosphorylation of all three subunits, in both the absence and presence of aldosterone. The increase in phosphorylation detected in our experiments could reflect a larger fraction of phosphorylated subunits or, alternatively, an increase in phosphate content per subunit. The change in phosphorylation correlates well with the increase in amiloride-sensitive sodium transport, suggesting that phosphorylation could be implicated in the control of sodium channel function in cells with endogenously expressed ENaC. The changes induced by the PKC inhibitor chelerythrine in the phosphorylation of all three ENaC subunits correlate well with changes in I_{Na} and are also consistent with the notion that phosphorylation of ENaC could determine the activity of the channel.

Previous studies have shown that application of vasopressin (6) and insulin (5, 10, 12, 13) to toad bladder and A6 cells pretreated with aldosterone results in a synergistic increase in sodium transport, rather than the additive effects of each agonist acting individually. This type of response is not observed, however, if the peptide agonist is administered only 5 h after the administration of aldosterone (12). These observations suggest that the mechanism of synergy is dependent on some factor that is required only after aldosterone reaches its maximum effect on sodium transport. It seems likely that that factor may be the abundance of ENaC located in the apical membrane. After the addition of aldosterone to A6 cells, an increased synthesis of xENaC subunits is initially observed after a latent period of ~3 h (1). Thereafter, the abundance of all three subunits increases in a gradual coordinated manner. In the present study the abundance of protein increased five- to eightfold after 16 h. Numerous observations have shown that sodium transport continues to rise after addition of aldosterone until it reaches a maximum between 12 and 24 h (7). Because aldosterone-induced sodium transport mirrors the abundance of ENaC in the apical membrane, these transport studies suggest that the highest levels of ENaC protein are achieved only after ~12 h of treatment with aldosterone. The corresponding activation of ENaC when insulin was applied to aldosterone-pretreated A6 cells or to control cells is shown in Fig. 4. In both conditions, a separate discernible increase in phosphorylation was observed when insulin was applied. In the case of the aldosterone-pretreated cells, however, the substrate for channel activation was larger than in control cells. This result is consistent with the notion that the magnitude of insulin-stimulated sodium transport is dependent on the size of the population of sodium channels available for activation and is, therefore, enhanced in aldosterone-pretreated cells because the abundance of protein is larger.

In conclusion, our results show that xENaC subunits are phosphorylated in the resting state in A6 cells and that stimulation of sodium transport by insulin is closely linked to increased phosphorylation, in both the absence and presence of aldosterone. The increase in the population of phosphorylated ENaC channels in the apical membrane could provide a means to augment sodium transport during the prolonged action of aldosterone and probably explains the synergistic increase in sodium transport when the action of insulin is superimposed. These experiments also strongly suggest that the posttranslational modification of the α-subunit that transforms the 85-kDa protein to one with an apparent molecular mass of 65 kDa,
confers the capacity for phosphorylation, which in turn might be necessary for the regulation of the channel in A6 cells.

ACKNOWLEDGMENTS

We acknowledge Lawrence J. Macala for expert help in performing the transport studies.

GRANTS

This work was supported by a Veterans Affairs Merit Award to J. P. Hayslett. D. Alvarez de la Rosa was supported by a research fellowship from the National Kidney Foundation.

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