Regulation of a Sodium Channel-associated G-protein by Aldosterone

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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. This channel is regulated, in part, by G-proteins, but it is not known if this mechanism is regulated by aldosterone. We report that aldosterone stimulates the expression of the 41-kDa α13 subunit of the heterotrimeric GTP-binding proteins in A-6 cells. Both mRNA and the total amount of this protein is increased by aldosterone. The G-protein is palmitoylated in response to the steroid, and the newly synthesized subunit is found to co-localize with the sodium channel. Aldosterone stimulation of sodium transport is significantly inhibited by inhibition of palmitoylation. These results suggest that aldosterone regulates sodium channel activity in epithelia through stimulation of the expression and post-translational targeting of a channel regulatory G-protein subunit.

Aldosterone increases epithelial sodium resorption in part by activating Na⁺ channels already present in the apical membrane (1, 2). The mechanism of steroid-induced activation of pre-existing channels is not known. Na⁺ channels of the type regulated by aldosterone have been shown in patch clamp studies to be gated by heterotrimeric G-proteins (3, 4), similar to other ion channels (5, 6), and the α13 subunit of the heterotrimeric G-proteins is known to be topographically localized with the channel (7). A number of G-protein α subunits have been shown to be post-translationally acylated, and these modifications promote membrane targeting and attachment (8–10). Although G-proteins are transcriptionally regulated in epithelial cells under conditions of growth and differentiation (11, 12), it is not known if they are synthesized, acylated, or targeted during stimulation of Na⁺ transport by steroids. We examined the possibility that aldosterone enhances association of the α13 G-protein with the Na⁺ channel by directing its synthesis and post-translational covalent lipid modification.

EXPERIMENTAL PROCEDURES

Cell Culture—A6 cells obtained from the American Type Culture Collection (Rockville, MD) were grown by limiting dilution and selected for use on the basis of high rates of amiloride-sensitive sodium transport. A6 cells are maintained in amphibian media with 10% fetal bovine serum in an atmosphere of humidified air-4% CO₂ at room temperature as described previously (13). All studies were carried out in cells grown on Millicell filters (Millipore, Bedford, MA). For electrophysiologic studies, A6 cells were grown on Millicell inserts and transepithelial potential difference and short circuit current were measured with a sterile, in-hood short-circuiting device as described previously (13). For biochemical studies, cells were grown on large Millicell filters (HAWP, 0.45 μm) attached to rings made from acrylic tubing. Membrane preparations were centrifuged at 1000 × g for 10 min to remove intact cells or fragments of filter, and the resulting supernatant represented whole cell lysate.

Protein Separation Methods—Proteins were precipitated overnight with 10 volumes acetone at −20 °C and collected by centrifugation. Precipitated proteins were dissolved in 200 μl of sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.025% bromphenol blue, pH 6.8) and heated at 100 °C for 5 min. Aliquots were removed for protein determination (DC protein assay, Bio-Rad) and for counting in a liquid scintillation spectrometer. Protein-matched samples were subjected to SDS-PAGE† using 5% stacking gels and 15% separating gels made using the buffer system of Laemmli (15). Molecular weight standards (Bio-Rad) were run in adjacent lanes. Gels were run at constant current (35 mA), stained with Coomassie Blue dye, or silver-stained (Silver Stain Plus Kit, Bio-Rad, Hercules, CA) impregnated with fluorographic enhancer (Enlightening, DuPont NEN) and dried under reduced pressure. Gels were exposed to X-Omat AR film (Eastman Kodak) for 14–30 days at −80 °C. Fluorograms were scanned with an EC 910 transmission densitometer for quantification.

Immunoprecipitation—Experiments were carried out on whole cell lysates. Acetone-precipitated pellets were solubilized in 1% Triton X-100, 50 mM Tris, pH 8. Four μl of rabbit polyclonal antibodies to either G-protein subunits (see below) or sodium channel antibodies were added to 200 μg of protein (50% protein assay, Bio-Rad, Hercules, CA) and incubated overnight at 4 °C. Then, 200 μl of washed (0.1 M phosphate buffer, pH 7) GammaBind Plus-coated Sepharose beads (Pharmacia) were added and incubated at room temperature on a rotary stirrer for an additional 2 h. The samples were then centrifuged briefly to sediment the beads, and the supernatant was removed and counted in a liquid scintillation spectrometer prior to discarding. The beads were washed in 500 μl of wash buffer (0.3% Nonidet P-40, 0.3% SDS, 0.9% NaCl, 50 mM Tris, pH 7.5). After a 5-min incubation with gentle agitation, at room temperature, the samples were centrifuged to sediment the beads, and the supernatant was removed and counted as before. Four additional washes were performed in an identical manner, and then the beads were washed an additional 2 times in 500 μl of deionized water. To the sedimented beads, 100 μl of sample buffer (2.5% SDS, 6 M urea, 5% 2-mercaptoethanol, 50 mM Tris, pH 6.8) was added and agitated at room temperature for 30 min. The beads were sedimented, and the supernatant was saved. This procedure was repeated once, and the two supernatants were pooled. 40 μl of glycerol and 10 μl

†The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
of bromophenol blue were added to the samples, and they were heated at 100°C for 5 min and then subjected to SDS-PAGE as described above.

Western Blotting—Equal amounts of solubilized protein were separated by SDS-PAGE as described above and transferred to nitrocellulose membranes in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol). Membranes were stained with Ponceau S (0.2%) (Ponceau S, 3% trichloroacetic acid, 3% sulfosalicylic acid). Non-specific binding sites in the transfers were blocked by a 1-h exposure to blocking solution (5% nonfat dried milk in Tris, pH 7.4). Transfers were overlaid with 1:1000 dilutions of specific antibody in blocking solution and incubated overnight at 4°C. The membrane was washed 3 times in phosphate-buffered saline with 0.025% Tween 20, pH 7.4. A horseradish peroxidase-conjugated secondary antibody, diluted 1:10,000 in 5% nonfat dry milk, 5% fetal bovine serum, in phosphate-buffered saline, pH 7.4, was allowed to incubate for 1 h at room temperature. The membrane was again washed, and signals were detected by an enhanced chemiluminescence system (Amersham) and exposed to Kodak X-OMAT AR film (Eastman Kodak Co.).

Northern Blotting—A6 cells were serum-depleted overnight and then exposed to 10^{-6} M aldosterone for 12–16 h. Poly(A) mRNA was isolated, and 5 μg of mRNA was electrophoresed through 1% formaldehyde-agarose gels. Gels were rinsed several times in RNA-free water prior to mRNA transfer to nitrocellulose. After transfer, the nitrocellulose filters were prehybridized at 42°C for 2 h. The prehybridization solution used is given in Sambrook et al. (19). A specific 5-BP-labeled α1 oligonucleotide probe was designed from conserved sequences for Gα3, showing least homology with Gα1 and Gα2 (CCTGGCAGCTTC-CCCAAA). This probe does not recognize α1 or α2 mRNA (data not shown). Approximately 500,000 cpm/ml 32P-labeled probe was added to the medium along with 10% dextran sulfate, and the filter was incubated at 42°C for 16 h. The filter was washed for 20 min at room temperature in 1× SSC, 0.1% SDS, followed by three washes of 20 min each at 68°C in 0.2× SSC, 0.1% SDS. Filters were exposed at −70°C for 48 h to Kodak X-Omat film. The autoradiograph for γ-actin mRNA was obtained by stripping the α1 probe and rehybridizing with the second probe to detect γ-actin, a housekeeping gene, for quantification. To control for variability, the Gα3 mRNA density values were normalized to the value for the γ-actin hybridization within each sample. Three different autoradiograph exposures were analyzed to ensure linearity. Three 150-mm filters of confluent A6 cells were used for mRNA isolation under each condition for each experiment.

Materials—G-protein antibodies raised to amino acid sequence Gα40–45 (GTNSNSGKSTIVKMK) part of the GTP binding domain (clone GA/1) (16) or to the carboxyl-terminal decapetide (KNNLKECGGLY) of α2 (EC/2) were purchased from DuPont NEN (7, 33). G-protein standards were purchased from Calbiochem. Polyclonal antibody to the sodium channel complex was prepared as described previously (17). All other chemicals were purchased from Sigma unless specifically indicated. [35S]Methionine, [3H]imrystic acid, and [14C]palmitic acid were purchased from DuPont NEN.

RESULTS

To determine if G-protein content was increased by aldosterone, A6 cells were labeled with [35S]methionine in the presence and absence of 0.1 μM aldosterone. Whole cell lysates were first incubated with an antibody directed against the common GTP binding site of G-proteins (GA/1), and the immunoprecipitated proteins were subjected to SDS-PAGE and autoradiography. Densitometry revealed that aldosterone enhanced metabolic labeling of a 41–45-kDa GTP-binding protein 3-fold compared to control (Fig. 1a). Other GTP-binding proteins were also labeled. To identify the protein labeled at 41 kDa, cell lysates were then subjected to immunoprecipitation with an affinity-purified antibody (EC/2) specific for Gα3 (DuPont NEN) (7, 16), directed against the carboxyl-terminal decapetide of α3, analyzed by SDS-PAGE and fluorography (Fig. 1b). Aldosterone enhanced metabolic labeling of Gα3 by 3–4-fold compared to controls. Next, whole cell lysates were subjected to Western blot analysis with EC/2 and visualized using peroxidase-conjugated secondary antibodies and chemiluminescence conjugates. Aldosterone increased the amount of the 41-kDa Gα3 G-protein 2–3-fold by densitometry (Fig. 1c).

To determine whether the increased expression of Gα3 was due to an increase in mRNA levels, A6 cells were serum-depleted overnight, then exposed to 0.1 μM aldosterone for 12–16 h, poly(A') mRNA was isolated, and Northern blot analysis was performed. The mRNA was probed with a specific Gα3 probe and a probe for γ-actin, a housekeeping gene (Fig. 2). Densitometry revealed that aldosterone induced a 1.6–2-fold specific increase in Gα3 message. These results indicate that aldosterone may increase Na+ transport via an increase in...
G-protein mRNA expression.

Experiments were designed to determine whether the newly synthesized G-protein becomes associated with the sodium channel. A6 cells were metabolically labeled with [35S]methionine in the presence and absence of 10^{-6} M aldosterone. Whole cell lysates were protein-matched and incubated with polyclonal antisera raised against a highly purified preparation of sodium channel isolated from bovine renal papilla (17) or pre-immune rabbit serum using the same conditions which had previously described the association of Ga_i3 with the sodium channel complex (7). Immunoprecipitated proteins were subjected to SDS-PAGE under reducing conditions and autoradiographed. As shown in Fig. 4, aldosterone stimulation of the Na^+ channel complex was regulated by aldosterone. This finding is consistent with previous electrophysiological and biochemical evidence that aldosterone acts primarily by activating pre-existing channels (1, 2). To determine whether post-translational modifications with lipids target the induced G-protein, we examined the effects of aldosterone on palmitoylation and myristoylation of Ga_i3. A6 cells were labeled with [1^{4}C]palmitate in the presence of 1 \mu M aldosterone or diluent. Cells were homogenized and crude membrane fraction was isolated by centrifugation at 100,000 \times g for 1 h and subjected to SDS-PAGE. As shown in Fig. 4a, aldosterone stimulated palmitoylation of several membrane proteins including a 41–45-kDa protein. There were also enhanced labeling of a broad band around 30 kDa, although palmitoylation of a smaller molecular mass protein at 18 kDa was not enhanced by aldosterone. This pattern of palmitoylation of membrane proteins was similar with both the 4- and 18-h exposure to aldosterone. In order to determine whether the 41–45-kDa palmitoylated protein was in fact a G-protein associated with the channel, we undertook immunoprecipitation of cellular proteins with G-protein and Na^+ channel antibodies following incubation with isotopically labeled acyl groups in the presence or absence of aldosterone. Whole cell lysates from A6 cells metabolically labeled with [3^{14}C]palmitate in the presence of 10^{-6} M aldosterone or diluent were subjected to immunoprecipitation with the sodium channel antibody. Immunoprecipitated proteins were resolved on 5–15% SDS-PAGE gels and visualized by autoradiography. Shown in a representative experiment of 4 experiments, A, immunoprecipitate from aldosterone-treated cells; C, immunoprecipitate from control cells. The last lane on the right labeled (−) represents whole cell lysates from metabolically labeled cells treated with aldosterone which were immuno-purified with preimmune rabbit serum. Numbers shown to the left demonstrate the molecular weight of the resolved channel subunits as determined from migration of molecular mass standards.
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in either the presence or absence of aldosterone, although other proteins were clearly labeled (data not shown). In A6 cells, it appears that the 41-kDa G-protein is palmitoylated but not myristoylated. A6 cells were next metabolically labeled with \([^{14}\text{C}]\)palmitate in the presence and absence of aldosterone, and whole cell lysates were subjected to immunoprecipitation with the Na\(^+\) channel antibody previously used to localize the G-protein to the channel (7). When protein-matched samples were immunoprecipitated with this antibody, aldosterone specifically increased palmitoylation of a 41-kDa protein (2–3-fold by densitometry) (Fig. 4c).

The physiological relevance of this observation was examined using an inhibitor of palmitoylation, 2-fluoropalmitic acid (18). 2-Fluoropalmitic acid (Biomol, Plymouth Meeting, PA) had no effect on basal sodium transport over short time courses at the concentration of 37.5 \(\mu\)M (Fig. 5), but markedly inhibited aldosterone-induced stimulation of sodium transport. In order to demonstrate that the action of this inhibitor might in fact be related to inhibition of G-protein palmitoylation, cells were metabolically labeled with \([^{14}\text{C}]\)palmitate in the presence and absence of aldosterone and 37.5 \(\mu\)M 2-fluoropalmitic acid. Protein-matched whole cell lysates were subjected to immunoprecipitation with GA/1 and subjected to SDS-PAGE and autoradiography. Fig. 6 demonstrates that aldosterone stimulates palmitoylation of several G-proteins including a band at 41 kDa and another around 30 kDa, and that labeling of these proteins with the acyl group is markedly inhibited by 2-fluoropalmitic acid.

**DISCUSSION**

The 41-kDa G\(_{\text{i3}}\) subunit has been shown previously to increase the open time of the apical sodium channel in excised apical membrane patches from A6 cells (3) and to be a component of the 700-kDa sodium channel complex (7). Our results demonstrate that aldosterone stimulates the expression of mRNA for this subunit and results in an increase in the total amount of this protein, which becomes associated with the sodium channel complex. Since this G-protein is thought to have a gating effect on the sodium channel, it seems reasonable to propose that increased expression and localization of this subunit may be one mechanism whereby aldosterone activates quiescent channels already present at or near the apical membrane (1). It also seems likely that aldosterone may direct a mechanism that localizes this G-protein to a site adjacent to the channel.

Several types of post-translational modifications have been described that are associated with membrane targeting or attachment of G-proteins. A number of G-protein \(\alpha\) subunits have been shown to be post-translationally acylated with either palmitate or myristate at sites near their amino termini, and these modifications promote membrane attachment (8–10). Smaller molecular weight G-proteins are targeted to membranes by a sequence of events involving highly conserved carboxyl-terminal cysteine residues. This pathway involves first prenylation of a cysteine residue, cleavage of the terminal amino acids, and subsequent carboxymethylation and/or acylation (20–22). Several previous observations suggest that these
targeting pathways might be involved in aldosterone action. First, aldosterone stimulates acylation-deacylation reactions (23), and inhibition of these reactions blocks both the transport response and the localization of aldosterone-induced proteins to membranes (24). Second, aldosterone stimulates carboxymethylation reactions which result in increased channel activation (25–27). The results presented here indicate that aldosterone stimulates localization of a 90–95-kDa membrane protein in A6 cells.

Taken together, the observations that aldosterone stimulates localization of a 41-kDa subunit of the channel and carboxymethylation of a 90–95-kDa subunit (30, 31), suggest the possibility that there may be more than one action of the channel. Such a suggestion has previously been made by Asher and Garty (30) on the basis of vesicle studies. They described an early stimulation of transport by aldosterone that was not stable to vesicle preparation and a prolonged effect that was stable to vesicle preparation. Carboxymethylation reactions of membrane-bound proteins are known to be reversible reactions due to the presence of methyl esterases in the membrane (31). We speculate that aldosterone may activate sodium channels already residing in the apical membrane, both through an early, reversible carboxymethylation and a later, more stable association of a gating G-protein.

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