Guanine nucleotide binding proteins in cultured renal epithelia: studies with pertussis toxin and aldosterone

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Sariban-Sohraby, Sarah, Michal Svoboda, and Frédérique Mies. Guanine nucleotide binding proteins in cultured renal epithelia: studies with pertussis toxin and aldosterone. Am. J. Physiol. 276 (Renal Physiol. 45): F10–F17, 1999.—The GTP-binding proteins from cultured A6 epithelia were examined in isolated membrane preparations. Binding of [35S]GTPγS revealed a class of binding sites with an apparent KD value of 100 nM and a Bmax of 220 pmol/mg protein. Short-term aldosterone treatment of the cells did not modify the binding kinetics, whereas pertussis toxin (PTX) decreased Bmax by 50%. The mRNA levels for Ga, Gb, Ga,o, and Gαs were not increased after aldosterone. The patterns of small M, G proteins and of PTX-ribosylated proteins were identical in membranes of both control and aldosterone-treated cells. Cross-linking of [α-32P]GTP, in control membranes, showed either no labeling or a faint band of M, 59.5 kDa. This protein become prominent after aldosterone, and its labeling decreased with spironolactone. Thus short-term aldosterone does not promote increased expression of known heterotrimeric G proteins in epithelial membranes but activates resident PTX-sensitive Gi proteins and stimulates the expression of a specific GTP-binding protein of M, 59.5 kDa.

A6 cells; photoaffinity labeling; sodium transport; GTP hydrolysis rate constant

EPITHELIAL SODIUM CHANNELS are regulated by aldosterone, although the molecular mechanisms are still largely uncharacterized. The basal activity of these channels can be modulated by GTP (9, 19) as well as by Ga, (6). When purified from A6 renal epithelial cells or bovine renal papillae, these Na+ channels comprise five to six polypeptides (3, 15), one of which, namely, a 90-kDa protein, is methylated after stimulating Na+ transport by short-term aldosterone (less than 2 h; Ref. 24). Methylation of this 90-kDa protein is stimulated in vivo by guanosine 5′-O-(3-thiotriphosphate) (GTPγS) in control membranes but not in membranes from cells exposed to aldosterone. Furthermore, aldosterone treatment of A6 cells results in a doubling of the rate of GTP hydrolysis by the isolated membranes (25). These observations support the idea that activation of G proteins mediates the early phase of aldosterone stimulation of apical Na+ permeability, possibly via methylation of the channels. Long-term exposure of A6 cells to aldosterone (16 h or more) is associated with increased metabolic labeling of the 41-kDa αi3 G protein in the apical membrane and with a 1.6- to 2-fold increase in the Ga, mRNA (22). The aim of the present study was to identify the various GTP-binding proteins associated with the isolated membranes and study the effect of pertussis toxin (PTX) and short-term aldosterone on the expression of these proteins with regard to increased GTPase activity. We detected several GTP-binding proteins in A6 membranes and showed that their levels of expression as well as mRNA were not modified by aldosterone. This indicates that the directional stimulation of the GTP hydrolysis rate is not linked to an increase in the membrane concentration of these G proteins and involves their activation through one or more additional regulatory steps. Interestingly, a novel 59.5-kDa GTP-binding protein was specifically expressed in the membranes of cells exposed to aldosterone. This protein may play a role in the reported G protein-mediated control of Na+ transport by aldosterone.

MATERIALS AND METHODS

Cell culture. A6 cells from Xenopus laevis toad kidney (American Type Culture Collection, Rockville, MD) were grown at 28°C in a humidified incubator gassed with 1% CO2 in air. Cultures were carried on plastic dishes and on porous supports as described previously (24). These supports were homemade 102-cm2 filter-bottomed cups that allowed the collection of large amounts of cells. The growth medium was Dulbecco’s modified Eagle’s medium (GIBCO) containing 75 mM NaCl and 8 mM NaHCO3 and supplemented with 5% fetal bovine serum (Hyclone, Logan, UT). When appropriate, cultures on porous supports were exposed to 100 nM aldosterone placed in the basolateral growth medium. Transepithelial measurements of voltage and resistance were performed on cells grown on 0.33-cm2 structures (Costar) using an EVOM voltohmmeter (World Precision Instruments) as described previously (25). The corresponding sodium current was calculated from these values obtained in the presence or absence of amiloride.

Membrane preparation. Cells grown to confluence on 102-cm2 porous supports were washed three times with ice-cold homogenization medium (HM) composed of 30 mM mannitol, 10 mM Tris-HEPES, and 10 mM MgCl2, pH 7.4, scraped in HM supplemented with phenylmethanesulfonyl fluoride (175 µg/ml), and homogenized with a Potter homogenizer (20 strokes). The homogenate was spun 15 min at 5,500 g, and the pellets were discarded. The supernatants were centrifuged 20 min at 43,000 g. The resulting pellets were resuspended in 100 mM mannitol and 10 mM Tris-HEPES, pH 7.4, and centrifuged once more at 43,000 g. The final pellets, enriched 10-fold in apical membranes (23), were resuspended and kept on ice or frozen at −80°C after snap-freezing in a dry-ice/ethanol slush.

Binding of [35S]GTPγS to A6 membranes. Binding of [35S]GTPγS to A6 membranes was assessed with the rapidfiltration technique described by Northup et al. (18). Ten micrograms of membrane protein were diluted in 140 µl of Tris·HCl, pH 8.0, 1 mM dithiothreitol (DTT), 100 mM NaCl,
and 30 mM MgCl₂ (buffer A) containing 0.025 µM [³²S]GTPγS (1.5 × 10⁶ cpm). Nonspecific binding was determined in the presence of 0.1 mM unlabeled GTPγS. Incubations were carried at 28°C for either 5 or 30 min, and the samples were then applied to 25-mm nitrocellulose filters (Millipore, HAWP 0.45) presoaked in buffer A. The filters were rapidly washed (under suction) with four successive 2-ml volumes of buffer A, oven-dried, dissolved, and counted in scintillation fluid (Instagel Plus, Packard). Specific binding was calculated as the difference in bound radioactivity in the absence or presence of 0.1 mM unlabeled GTPγS. Blank values were 211 ± 31 cpm (n = 28), i.e., 0.14% of the applied radioactivity. This filtration method showed a linear increase in binding with increasing protein concentration in the range tested (5–41 µg/sample). Binding was linear with time up to 10 min. Initial rates and equilibrium binding were thus measured, respectively, after 5 and 30 min of incubation with the radioactive ligand.

RT-PCR. Total RNA was extracted from control and aldosterone-treated A6 cells using RNA Nosi (Biogentex). The RNA samples were treated with DNase I and reverse transcribed with Superscript II (GIBCO-BRL) using random primers. cDNAs were amplified using primers with Superscript II (GIBCO-BRL) using random primers at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min (30 cycles), with a 10-min extension at 72°C at the end of the cycling. The PCR reaction products were resolved on 1.5% agarose gels in 0.5 TBE buffer (10 mM Tris, 10 mM Na₂HPO₄, pH 7.0, 45 mM NaCl) for 2 h. The gels were dried, autoradiographed, and relative absorptions were quantified with a BIO-1D video-image analysis system (Viberward 658–678; reverse 894–874; GenBank accession no. X56099). Gα₂(agr) (forward 447–467; reverse 868–633; no. X56090), Gα₅(agr) (forward 606–627; reverse 837–816; no. X14366), and Gα₂ (forward 528–549; reverse 764–743; no. U10502). Glucose-6-phosphate dehydrogenase (GPDH) was used as control (forward 564–584; reverse 1015–996; no. U41753). As a positive control of aldosterone effect on mRNA levels, we used primers designed from ASUR 1, a cloned DNA sequence from A6 cells kindly provided by F. Verrey. This mRNA was shown to increase 400–500% after short-term aldosterone treatment (27). Primers were as follows: ASUR 1 forward, GTA CCC AGG TCAAGG GTC AA; and ASUR 1 reverse, ACT GTA CCC AGG TCAAGG GTC AA; and ASUR 1 reverse, ACT

Binding of [α-³²P]GTP on Western blots. Membrane proteins separated by SDS-PAGE (12% polyacrylamide gels) were transferred to nitrocellulose paper in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) using the Trans-Blot apparatus from Bio-Rad (30 V overnight). Blots were incubated for 30 min at room temperature with [α-³²P]GTP (1 µCi/ml, 0.1 mCi/cm²) in binding buffer (50 mM Tris·HCl, pH 7.5, 0.3% Tween 20, 5 mM MgCl₂, and 1 mM EGTA) with or without 1 µM unlabeled GTP. Blots were then rinsed with seven to eight changes of binding solution over 1 h and air dried. Bound radioactivity was detected by autoradiography.

Photoaffinity labeling of membrane proteins. The UV-mediated cross-linking of [α-³²P]GTP to membrane proteins was adapted from Basu and Modak (2). The incubation mixture, in a total volume of 55 µL, contained 20 mM Tris·HCl, pH 7.4, 1 mM ATP, 1 mM DTT, 100 mM NaCl, 30 mM MgCl₂, 0.1% Lubrol, 0.25 µM [α-³²P]GTP (800 Ci/mmol), and 100 µg protein. After a 10-min incubation on ice, the samples were exposed to broad-spectrum UV light for 7 min at a distance of 15 cm and at 4°C. The reaction was stopped by the addition of 20 µL of 2 mM GTP. Samples were resuspended in sample buffer (70 mM Tris·HCl, pH 6.8, 2% SDS, 12.5% glycerol, 0.02% bromophenol blue, and 5% β-mercaptoethanol) and separated on 12% polyacrylamide gels by SDS-PAGE in a Bio-Rad Protean II gel apparatus. Bound radioactivity was detected by autoradiography.

Isotopes were obtained from New England Nuclear, and PTX was from Sigma. Gel electrophoresis reagents and standards were from Bio-Rad. Proteins were measured using the BCA Protein Assay kit from Pierce (Rockford, IL). Antibodies against the α-subunits of G₁, G₂, G₃, and G₄ proteins were from Calbiochem (San Diego, CA).

RESULTS

In previous studies, we observed an increase of both the sodium transport rate and GTPase activity in membranes isolated from A6 cells exposed to aldosterone (25). It was possible that the increase in activity was due simply to an increase in the number of hydrolysis sites. We tested this directly in the present studies. Experiments were carried out in vitro on membrane preparations which are enriched 10-fold in apical markers (23).

Equilibrium binding of [³²S]GTPγS to A6 membranes. Binding of [³²S]GTPγS was measured in membranes prepared from control cells and from cells exposed to 100 nM aldosterone for 4 h. In the presence of MgCl₂, binding was linear up to 10 min and reached equilibrium after 30 min at 28°C. Binding was a
A saturable function of GTPγS concentration with similar characteristics in membranes from control and aldosterone-treated tissues (Fig. 1A). The membranes were enriched 7- to 10-fold in binding sites compared with their respective cell homogenates (n = 3). Linearization of the binding data according to Scatchard is shown in Fig. 1B. The values of $K_d$ and $B_{\text{max}}$ summarized in Table 1, are not significantly different for control and aldosterone-treated tissues (unpaired $t$-test). Likewise, the initial rate of binding (1.24 ± 0.08 pmol·min$^{-1}$·mg protein$^{-1}$) was unmodified after aldosterone (1.20 ± 0.05 pmol·min$^{-1}$·mg protein$^{-1}$). Both GTP and GDP behaved as competing nucleotides for the binding of GTPγS with an EC$_{50}$ value of 1 µM for GTP and 5 µM for GDP (n = 2).

Clearly, the total number of GTPγS binding sites was not changed by aldosterone. These studies were pursued on tissues treated with PTX. This toxin specifically ADP-ribosylates Goi proteins and was found to inhibit both the aldosterone-stimulated GTPase activity and sodium transport (25).

After incubating the membranes with activated toxin, equilibrium binding was inhibited by 50.4 ± 4% (n = 12) in control membranes and 53 ± 5% (n = 12) in membranes from aldosterone-treated cells (Fig. 2). In both conditions, the inhibition by PTX was related to a decrease in $B_{\text{max}}$ with little change in affinity (Fig. 1B; Table 1). From the binding data and the values of GTPase activity, we calculated rate constants for GTP hydrolysis, $k_{\text{cat}}$, GTP (moles of GTP hydrolyzed/number of binding sites) after aldosterone and/or PTX (Table 2).1

Table 1. Maximum rates of binding and half-saturation constants obtained after linearization of the data as presented in Fig. 1B

<table>
<thead>
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<th>$K_d$, nM</th>
<th>$B_{\text{max}}$, pmol/mg protein</th>
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<tbody>
<tr>
<td>Control</td>
<td>91 ± 0.8</td>
<td>233 ± 29</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>100 ± 0.9</td>
<td>206 ± 12</td>
</tr>
<tr>
<td>Control + PTX</td>
<td>87 ± 1.1</td>
<td>116.9 ± 15</td>
</tr>
<tr>
<td>Aldosterone + PTX</td>
<td>91 ± 0.6</td>
<td>110.4 ± 11</td>
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Values are means ± SE. $B_{\text{max}}$, maximum rate of binding; $K_d$, half-saturation constant. PTX, pertussis toxin.

1 Values of GTPase activities are taken from Ref. 25 and have been verified in this study.
Clearly, the PTX-sensitive component of \( k_{\text{cat}} \text{GTP} \) after aldosterone was increased 13-fold over control. Based on the assumption that PTX-sensitive binding reflects the membrane concentration of \( G_\alpha \) proteins, this supports the idea that aldosterone stimulates the activity and not the abundance of this important \( G \) protein.

It is possible that the observed similarity of the proportional decrease in binding sites after PTX was due to the instability (i.e., rapid turnover) of sites in the membrane. This was tested directly with cycloheximide, which inhibits \( \text{Na}^+ \) transport but not GTP hydrolysis (8). As shown in Fig. 3, in tissues exposed to cycloheximide, inhibition of transepithelial \( \text{Na}^+ \) current was measurable after 2 h and aldosterone stimulation was completely prevented, but no change in initial rates of binding was observed up to 24 h in either control or aldosterone-treated cells. Thus we cannot attribute the decrease in binding after PTX to a nonspecific, time-dependent loss of sites.

Because binding studies measure total membrane concentration of sites, they do not exclude the existence of a minor population of \( G \) proteins specifically induced after aldosterone. Therefore, we verified by RT-PCR and by the more sensitive RNase protection assay (RPA) method the level of mRNA for the \( \alpha \) subunits of various heterotrimeric \( G \) proteins.

RT-PCR and RPA. mRNA abundance was estimated by quantitative RT-PCR in identical amounts of total RNA from cells grown in the absence or presence of 100 nM aldosterone.

The results of the amplification of \( G_\alpha_{i-3} \) are shown in Fig. 4. We did not observe any increase in \( G_\alpha_{i-3} \) mRNA with aldosterone up to 2 h (Fig. 4A) or 24 h (data not shown). This is in contrast to the increase in the positive control ASUR 1 (27), which doubled after 30 min (Fig. 4B). Likewise, \( G_\alpha_{i-1}, G_\alpha_{o}, G_\alpha_{q}, \) and \( G_\alpha_{s} \)Table 2. Rate constants of GTP hydrolysis calculated from GTPase and binding activities in absence and presence of PTX.

<table>
<thead>
<tr>
<th></th>
<th>GTPase Activity, pmol·min(^{-1})·mg(^{-1})</th>
<th>( B_{\text{max}} ), pmol/mg</th>
<th>( k_{\text{cat}} \text{GTP}, \text{min}^{-1} )</th>
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<tr>
<td><strong>Total sites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15.1 ± 0.4</td>
<td>232 ± 29</td>
<td>0.065 ± 0.009</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>34.2 ± 0.96*</td>
<td>206 ± 12</td>
<td>0.163 ± 0.013*</td>
</tr>
<tr>
<td><strong>PTX-sensitive sites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.48 ± 0.21</td>
<td>116.1 ± 14.6</td>
<td>0.013 ± 0.003</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>17.0 ± 0.51*</td>
<td>95.6 ± 5.56</td>
<td>0.177 ± 0.015*</td>
</tr>
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</table>

Values are means ± SE. Control and aldosterone-treated tissues are compared. *Statistically significant (unpaired t-test). Sensitive sites are calculated as the difference between total number of sites and sites measurable in the presence of 1 µg/ml of toxin. \( k_{\text{cat}} \text{GTP}, \text{rate constant of GTP hydrolysis.} \)

Fig. 3. Effect of cycloheximide on transepithelial \( \text{Na}^+ \) current and on \[^{35}\text{S}]\text{GTP}_{\gamma} \text{S} \) binding. A6 cells grown to confluence on porous supports were exposed to 100 nM aldosterone (■), 1 µg/ml cycloheximide (▲), or 1 µg/ml cycloheximide plus 100 nM aldosterone (●) for the times indicated. \( \text{Na}^+ \) currents of control tissues were 12.3 ± 0.16 µA/cm\(^2\) at time 0 and stayed stable over 24 h. Calculated amiloride-sensitive \( \text{Na}^+ \) currents are shown. Initial rates of \[^{35}\text{S}]\text{GTP}_{\gamma} \text{S} \) binding, in pmol·min\(^{-1}\)·mg protein\(^{-1}\), are also shown (●). Values of binding obtained in control and aldosterone-treated tissues were indistinguishable (n = 6).

Fig. 4. RT-PCR amplification (30 cycles) of \( G_\alpha_{i-3} \) (A) and ASUR 1 (B). Total RNA was extracted from A6 cells grown in absence or in presence of 100 nM aldosterone. Results are shown for incubation times with aldosterone up to 120 min. Fluorescent amplified DNA fragments appear at the top of each graph. These are representative of 3 experiments. AU, absorbance units.
mRNAs remained unchanged (data not shown). The RT-PCR results were confirmed by RPA as shown in Fig. 5. Aldosterone did not modify the signal for Ga\textsubscript{i-3} compared with ASUR 1 (positive control) and G\textsubscript{3PDH} (negative control). These data rule out the existence of even a small increase in mRNA which would have been unnoticed by PCR.

We also visualized various GTP-binding proteins by biochemical approaches that classically target either heterotrimeric or small Mr G proteins.

**Binding of [\textalpha-\textsuperscript{32P}]GTP on Western blots.** Low-molecular-weight G proteins uniquely renature upon Western blotting and bind GTP specifically. A6 membrane proteins were separated by SDS-PAGE and transferred to nitrocellulose. Exposure of these blots to [\textalpha-\textsuperscript{32P}]GTP resulted in the specific labeling of a group of proteins in the Mr range of 26–29 kDa as well as a 21-kDa polypeptide (Fig. 6). The same pattern was seen in membranes from control (lane 1) or aldosterone-treated cells (lane 2). When \textsuperscript{35S}GTP\textsubscript{yS} was used instead of [\textalpha-\textsuperscript{32P}]GTP, we observed specific labeling of a 26-kDa polypeptide only (data not shown). Because aldosterone did not alter the expression or the pattern of these small G proteins, they were not characterized further (see DISCUSSION).

**ADP-ribosylation of A6 membranes.** Since PTX-sensitive sites are clearly important in the response of cells to aldosterone (see above), it was of interest to identify the G proteins targeted by the toxin. Exposure of A6 membranes to \textsuperscript{32P}NAD in the presence of PTX, followed by gel electrophoresis resulted in the labeling of a single band of Mr 41 kDa in control membranes (Fig. 7, lane 1). After aldosterone, the amount of

![Fig. 5. RNase protection assays (RPA). RPAs of glucose-6-phosphate dehydrogenase (G\textsubscript{3PDH}) (group 1), ASUR 1 (group 2), and Ga\textsubscript{i-3} mRNA (group 3) were performed using total RNA from control A6 cells (solid bars) or from cells treated with aldosterone for 2.5 h (hatched bars) or 24 h (open bars). Autoradiograms were analyzed with a BIO-1D video-image analysis system as described in MATERIALS AND METHODS. Relative absorption (AU, absorbance units) of protected fragments is shown, representative of 3 experiments.](image)

![Fig. 6. Labeling of low-molecular-weight G proteins in A6 membranes. Autoradiogram of a Western blot of membranes from control (lane 1) and aldosterone-treated cells (lane 2) separated on 12% polyacrylamide gels. Each lane was loaded with 80 µg protein. Blots were exposed to 12.5 nM [\textalpha-\textsuperscript{32P}]GTP for 90 min, and nonspecific binding was examined in presence of 1 µM cold GTP (lanes 3 and 4). Films were exposed for 2 wk at –80°C. Background shown is the best we could obtain even after extensive rinsing of the blots. Molecular masses of the standards are indicated on left.](image)

![Fig. 7. ADP-ribosylation of A6 membrane proteins. Autoradiogram of A6 membrane proteins (40 µg/lane) separated on 8% polyacrylamide gels after a 30-min exposure of [\textalpha-\textsuperscript{32P}]NAD and PTX (lanes 1 and 3 for control membranes and membranes of cells treated with aldosterone for 4 h, respectively). In absence of toxin, label incorporation was not observed (lane 2). Molecular masses of the standards are indicated on left. Films were exposed for 2 days at –80°C.](image)
ADP-ribosylated protein remained unchanged (lane 3). This 41-kDa polypeptide was recognized by anti-Gα3 antibodies on Western blots (data not shown; see Ref. 25). Unlike a previous report by Ausiello et al. (1), we did not observe ADP-ribosylation of other proteins associated with the Na+ channels such as the 90- to 95-kDa polypeptide (1).

Photoaffinity labeling of membrane proteins. The GTP binding sites may be modified by SDS solubilization. Therefore, we used photoaffinity covalent labeling to examine the binding sites in their native form. The results of photoincorporation of high specific activity [α-32P]GTP (0.25 µM) into membrane proteins are shown in Fig. 8. In the absence of UV irradiation, there was no covalent binding of label. After UV irradiation of control membranes (Fig. 8A, lane 1), little or no incorporation was observed. After aldosterone, however, intense labeling of a 59.5-kDa band was observed (Fig. 8A, lane 2). Photoincorporation of the label was completely blocked with 100 µM GTP (Fig. 8A, lane 3) but decreased only 16% with 100 µM ATP (Fig. 8B, left lane) as measured by densitometry scanning of the autoradiograms (n = 2). This 59.5-kDa protein was probed on Western blots by commercial antibodies against either Gαi3, Gaia, Gαs, or Gαq, but no labeling was observed (data not shown). Furthermore, [32P]NAD ribosylation of this protein in the presence of PTX did not occur (Fig. 7). However, labeling of the 59.5-kDa protein was decreased in membranes from cells exposed to both aldosterone and spironolactone, a competitive antagonist of the hormone, suggesting that this membrane protein is specifically induced by aldosterone (Fig. 8C).

DISCUSSION

Evidence has accumulated that G proteins play significant roles in controlling the activity of amiloride-inhibitable Na+ channels at the apical membrane of epithelia (see Ref. 26 for review) as well as in lymphocytes, which also possess these channels (5). However, the G protein-mediated pathway(s) activated by aldosterone in the control of Na+ channels is clearly different from the ones involved with membrane-bound G protein-coupled receptors and diffusible second messengers (see Ref. 28 for a review).

Activation of Na+ channels located in the apical membrane of responsive epithelia by aldosterone causes an increase in the Na+ transport rate. This results from an increase of the channel’s open probability, the number of functional channels, or both (11, 14). In sodium-reabsorbing epithelia, there are no cell-surface receptors for aldosterone. This delays the effect of the hormone on the Na+ transport rate, which probably involves the synthesis of a number of proteins, many of which, still have not been identified. GTP-dependent methylation of the apical Na+ channels appears to mediate the short-term effects of aldosterone on apical Na+ permeability (13, 24, 26).

Because aldosterone activates a specific membrane GTPase, we examined the G proteins that reside in the

Fig. 8. Photoaffinity labeling of A6 membrane proteins. A: membranes (100 µg per sample) were incubated with 0.25 µM [α-32P]GTP in presence and absence of an excess unlabeled GTP and exposed to broad-spectrum UV light as described in MATERIALS AND METHODS. Membrane proteins were separated on 12% polyacrylamide gels and the gels were exposed to Hyperfilm-MP (Amersham, Belgium) for 1 wk at −80°C. Molecular mass marker positions are as follows, from top to bottom: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Lane 1, control membranes; lane 2, membranes from cells treated with aldosterone for 4 h; lane 3, + 100 µM GTP. Photograph is representative of 3 experiments. B: photoaffinity labeling of membranes from cells treated with aldosterone (right lane) and exposed to 100 µM ATP during the irradiation period (left lane). C: photoaffinity labeling of membranes from cells treated with aldosterone (left lane) or with aldosterone and 10 µM spironolactone for 4 h (right lane). For B and C, molecular mass marker positions are the same as in A (first 4 markers, from 97 to 31 kDa). Exactly 100 µg of protein were loaded in each lane (n = 2).
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cell membrane before and after hormone treatment. Our membrane preparations are enriched 10-fold in apical markers as well as in GTP-γS binding sites, but we cannot exclude the presence of basolateral membranes.

Using techniques that target different G proteins, we identified the following three groups of specific resident GTP-binding proteins: 1) several small G proteins of molecular mass 21 and 26–29 kDa (the pattern as well as the level of expression of these proteins did not change after aldosterone); 2) a 41-kDa protein that was ADP-ribosylated by PTX, recognized by an anti-Gi-3 antibody, and also not modified by aldosterone; and 3) a 59.5-kDa protein labeled by photoaffinity whose expression at the cell membrane was triggered by treating cells with aldosterone. This protein has not been described previously. Its labeling was blocked by an excess of unlabeled GTP but not by ATP. The protein was not recognized by antibodies directed against the α-subunits of Gi-3, Go13, Goi or Go or by ADP ribosylation with PTX. Spiro nous lactone strongly diminished the protein labeling.

We also observed the binding of [35S]GTP-γS to apical membrane preparations was not altered after 4 h of aldosterone in terms of the kinetics (initial rate and equilibrium binding), the affinity for the ligand, or the number of binding sites. Likewise, using two independent approaches, we did not observe any increase in mRNA levels for the α-subunits of Gi-3, Go13, Goi, or Go in A6 cells exposed to aldosterone for either 4 or 24 h. Rokaw et al. (22), using the Northern blot technique, have reported an increase in the level of Gi-3 mRNA in A6 cells after 16 h of aldosterone. However, this increase was relatively small (only 1.6- to 2-fold) and may not be physiologically relevant to the early, 4-h phase of the aldosterone response.

The action of PTX in A6 cells appears complex. Several reports have indicated that the sensitivity of Na+ channels to PTX is modulated by the biochemical state in which they reside. For example, their level of phosphorylation/dephosphorylation may influence the channel’s response to GTP-γS (5, 7, 12, 19). In the present study, equilibrium binding of GTP-γS to both control and aldosterone-treated tissues was inhibited by 50% in the presence of PTX. Previously, we observed similar decreases in the rates of Na+ transport and GTP hydrolysis in aldosterone-treated tissues exposed to PTX (25). Since this toxin is a specific marker of Gi-3, this suggests that half of the GTP binding sites are involved in the toxin-sensitive stimulation of GTP hydrolysis following aldosterone. In control membranes, we also observed a 50% decrease in GTP-γS binding after PTX treatment. However, the toxin has only small effects (~15%) on basal Na+ transport and GTP hydrolysis (25). This indicates that under basal conditions, Gi is present in the membrane but contributes little to the control of steady-state Na+ transport. In support of this idea, we observed a dissociation of GTP binding from Na+ transport after cycloheximide treatment. This also indicates that the GTP binding sites and the sodium channels have different residence times in the cell membrane and suggests that their expression is independently regulated.

After aldosterone, the increase in the rate constant of GTP hydrolysis results essentially from the activation of PTX-sensitive G proteins. The kcat,GTP values obtained in this study are lower than the values found in nonepithelial tissues equipped with soluble signaling proteins (17). Values of kcat,GTP in epithelia are not available in the literature for comparison. The large increase (over 13-fold) in the rate of PTX-sensitive GTPase activity after aldosterone associated with a constant membrane concentration of Gi-3 proteins points to the activation of these proteins by an additional regulatory component such as a member of the RGS (“regulators of G protein signaling”) or the GAP (“GTPase activating proteins”) families (4, 7, 16, 17). The basal, unstimulated PTX-insensitive GTPase activity could be related to the presence of the low-molecular-weight G proteins. In this regard, subcellular localization of G proteins to the apical membrane of epithelia has been reported for low-molecular-weight G proteins (20). One of these proteins, a 29-kDa polypeptide located in the apical membrane of collecting duct cells of mammalian kidney, was identified as ras (11), but no specific function has been assigned to it or to any other small G proteins in epithelia. Recently, Spindler et al. (27) identified in A6 cells an early adrenal-steroid-upregulated RNA (ASUR 5) as the A transcript of Xenopus K-ras2. Its role in the stimulation of sodium transport by aldosterone has not been reported.

In summary, we found that short-term aldosterone does not promote the expression of classic α-subunits of heterotrimeric G proteins in A6 cells but stimulates the GTP hydrolysis rate by activating resident PTX-sensitive G proteins. Aldosterone also increases specifically the expression of a novel 59.5-kDa GTP-binding protein, the role of which, as a regulatory component in the complex cellular response to aldosterone, remains to be established.

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