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Purified Epithelial Na^+ Channel Complex Contains the Pertussis Toxin-sensitive $G\alpha_{i-3}$ Protein*

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We have recently demonstrated that the amiloride-sensitive Na^+ channel in the apical membrane of the renal epithelial cell line, A6, is modulated by the α_{i-3} subunit of the G_{i-3} protein. We also showed that a 700-kDa protein complex can be purified from the membranes of A6 epithelia which (*a*) can reconstitute the amiloride-sensitive Na^+ influx in liposomes and planar bilayer membranes and (*b*) consists of six major protein bands observed on reducing sodium dodecyl sulfate-polyacrylamide gels with molecular masses ranging from 35 to 320 kDa. The present study was undertaken to determine if the α_{i-3} subunit was a member of this Na^+ channel complex. $G\alpha_i$ structure and function were identified by Western blotting with specific $G\alpha_i$ subunit antibodies and Na^+ channel antibodies, through ADP-ribosylation with pertussis toxin, and by immunocytochemical localization of the Na^+ channel and $G\alpha_i$ proteins. We demonstrate that two protein substrates are ADP-ribosylated in the 700-kDa complex in the presence of pertussis toxin and are specifically immunoprecipitated with an anti-Na^+ channel polyclonal antibody. One of these substrates, a 41-kDa protein, was identified as the α_{i-3} subunit of the G_{i-3} protein on Western blots with specific antibodies. Na^+ channel antibodies do not recognize $G\alpha_{i-3}$ on Western blots of Golgi membranes which contain α_{i-3} but not Na^+ channel proteins, nor do they immunoprecipitate α_{i-3} from solubilized Golgi membranes; however, α_{i-3} is coprecipitated as part of the Na^+ channel complex from A6 cell membranes by polyclonal Na^+ channel antibodies. Both α_{i-3} and the Na^+ channel have been localized in A6 cells by confocal imaging and immunofluoresence with specific antibodies and are found to be in distinct but adjacent domains of the apical cell surface. In functional studies, α_{i-3}, but not α_{i-2}, stimulates Na^+ channel activity. These data are therefore consistent with the localization of Na^+ channel activity and modulatory α_{i-3} protein at the apical plasma membrane, which together represent a specific signal transduction pathway for ion channel regulation.

The regulation of Na^+ transport in renal epithelia is in large part a function of the modulation of Na^+ channel activity in the apical membrane. In a number of epithelia, including cultured toad kidney A6 cells, Na^+ channel activity has been shown to be influenced by classical hormone signal transduction pathways at the basolateral membrane involving biochemical signaling through protein kinase activity (1–5). Recent patch-clamp data from our laboratory have demonstrated that the Na^+ channel is activated in excised, pertussis toxin-treated apical membrane patches from A6 cells by addition of the α subunit of the G_{i-3} protein[1] (6). These studies were further supported by the observation that guanine nucleotides regulate the apical Na^+ channel, thus providing functional evidence for the existence of an endogenous G_i protein/Na^+ channel complex localized in the apical membrane of the A6 epithelia. This G_{i-3} protein is topographically distinct from receptor-coupled Gs and G_{i-2} proteins traditionally localized in the basolateral membrane which are responsible for the generation of second messengers (7). $G\alpha_{i-3}$ is localized predominantly on the Golgi membranes in epithelial cells (7, 8). In this report we demonstrate that the α_{i-3} subunit is also a structural component of the 700-kDa Na^+ channel complex in the apical membrane of A6 epithelial cells. We show that the Na^+ channel complex contains the α_{i-3} 41-kDa protein, which is identified using specific α_{i-3} peptide antibodies and is a substrate for ADP-ribosylation by pertussis toxin. The α_{i-3} subunit was localized by confocal imaging and was found to be concentrated in a domain in the apical membrane adjacent to the Na^+ channel in A6 cells. The functional significance of this localization is supported by the demonstration that α_{i-3}, but not α_{i-2}, regulates Na^+ channel activity as assessed by the patch-clamp technique. These data indicate that the Na^+ channel complex contains the α_{i-3} regulatory protein, which is functionally linked to apical Na^+ channel regulation.

EXPERIMENTAL PROCEDURES

Na^+ Channel Purification—The procedures for the isolation and purification of the amiloride-sensitive Na^+ channel from amphibian A6 cultured cells are detailed in Sariban-Sohraby and Benos (9) and Benos *et al.* (10) and were used here without modification.

Cell Culture—Clonal A6 cells (ATCC no. CCL102) derived from *Xenopus laevis* renal tubular cells were used between passages 20–36. The procedures for growing the A6 cells on permeable supports (Nucleopore or Millipore HAWP, 0.45-μm filters) are detailed in earlier reports (6, 10). Cells utilized for patch-clamp studies and

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[1] The abbreviations used are: G protein, signal-transducing, nucleotide-binding protein of subunit structure $\alpha\beta\gamma$; $G\alpha_i$ or $\alpha_{i-1,2,3}$, the α subunit family of pertussis toxin-sensitive G proteins responsible for inhibition of adenylyl cyclase and modulation of ion channels; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine 5-(3-*O*-thio)triphosphate; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; HPLC, high performance liquid chromatography.

immunofluorescence were cultured on glass coverslips in tissue culture medium as previously described (6, 7). Briefly, cells were grown in a Coon's modification of Ham's F-12 and Liebovitz's F-15 media modified to contain 105 mM NaCl and 25 mM $NaHCO_3$. The mixture was supplemented with 10% fetal bovine serum (GIBCO). Cells were maintained at 27 °C in an atmosphere of humidified air plus 5% CO_2.

Antibodies—Polyclonal antisera raised against a highly purified preparation of sodium channel from bovine renal papilla were used for immunoprecipitation, Western blotting, and immunolocalization of the Na^+ channel (11, 12). Antisera from different rabbits show specificity for the epithelial Na^+ channel as assayed by enzyme-linked immunoabsorbent assay, immunodot blots, Western blots, and by immunoprecipitation of iodinated channel proteins from a heterogeneous mixture of solubilized kidney membrane proteins. All antisera give the same staining pattern for the Na^+ channel by immunofluorescence (see Fig. 1*A*). Antisera from different rabbits all immunoprecipitate the complex of proteins described previously (~300 to ~30 kDa) (11) (Fig. 4*A*), but each antiserum recognizes only two or three of these proteins by Western blotting. Thus, the antiserum used in the present study recognizes only proteins at 320, 150, and 95 kDa by Western blotting (see Fig. 4*B*).

Polyclonal rabbit antibodies raised against peptides of α_i subunits were the kind gift of Dr. A. Spiegel (National Institutes of Health). An affinity-purified antibody (EC-2), which was raised against a carboxyl-terminal decapeptide of α_{i-3}, was used to specifically identify α_{i-3} on Western blots (13) and for immunolocalization of α_{i-3} in LLC-PK_1 cells and other cells, as previously described (7, 8).

Patch-Clamp Measurement of Na^+ Channel Activity—The patch-clamp technique was carried out as we have previously described (6). Data were obtained solely from excised inside-out patches. Holding potentials were between +60 and +80 mV. Patch-pipette solution was (in mM); 115 NaCl, 5 KCl, 0.8 $MgSO_4$, 1.2 $CaCl_2$, and 10 Hepes, pH 7.4. The perfusion solution was a modification of the patch pipette solution where Na^+ was replaced in equimolar concentration with K^+, thus containing 115 mM KCl and 5 mM NaCl, with all other solutes remaining the same. Data are presented as percent of total open time, *i.e.* $100 \times p_o$ (p_o = open probability). Data are expressed as mean ± S.E. for a minimum of three separate channels. Open probability did not statistically change at a given holding potential for up to 15 min. All additions were made to the cytosolic bathing solution. Two microliters of activated pertussis toxin (Peninsula Laboratories, Belmont, CA) containing 10 mM dithiothreitol and 1 mM NAD^+ were added to the bathing solution (0.4 ml) to a final concentration of 100 ng/ml as previously described (6). Human α_{i-2} and α_{i-3} were activated with GTPγS and purified by ion-exchange chromatography (6, 14). Activated α_{i-3} or α_{i-2} were added to the bathing solution with an albumin-coated pipette dispenser to a final concentration of 20 pM, as previously described (6).

Gel Electrophoresis and Western Blotting—Samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 50 mM Tris-HCl buffer (pH 6.8) containing 2.5% sodium dodecyl sulfate, with 13 mM dithiothreitol, and boiled for at least 2 min before loading onto 8% polyacrylamide gels. HPLC-purified Na^+ channel proteins from A6 cell membranes were electrophoresed and transferred to nitrocellulose in transfer buffer (25 mM Tris, 20% methanol, 192 mM glycine, pH 8.3) for Western blotting. Golgi membranes, which are enriched in the $G\alpha_{i-3}$ subunit, were isolated from rat liver homogenates by standard procedures as previously described (8, 15) and electrophoresed on 10% polyacrylamide gels prior to transfer onto Immobilon membrane for Western blotting. Nonspecific protein binding sites on the transfers were blocked by a 1-h exposure to 5% nonfat dry milk in Tris buffer, pH 7.4, and transfers were then overlayed with 1:1000 dilutions of specific antiserum and incubated overnight at 4 °C. After Tris-Tween saline washes (20 mM Tris, 500 mM NaCl, 0.5% Tween 20, pH 7.5) , the transfers were incubated for 1 h in a solution of goat anti-rabbit IgG antibody linked to alkaline phosphatase followed by nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate/Tris buffer for color development, or bound antibodies were detected with ^{125}I-protein A followed by autoradiography.

Pertussis Toxin-induced ADP-ribosylation—Apical membranes obtained from A6 cells grown on filters were solubilized (9), followed by pertussis toxin-induced ADP-ribosylation, which was carried out as previously described (5) in a buffer containing 10 mM Tris base (pH 7.5), 1 mM EDTA, 5% β-mercaptoethanol, 1 mM ATP, and 0.01 mM GTP. The assay was started by the addition of [^{32}P]NAD to a final concentration of 10 μM; the solubilized A6, Na^+ channel protein complex was incubated for 45 min at 32 °C. The samples were spun for 2 h at 6000 rpm followed by boiling for 3 min in sodium dodecyl sulfate and electrophoresis. Isolated rat liver Golgi membranes were incubated with pertussis toxin for ADP-ribosylation of the $G\alpha_{i-3}$ subunit as described previously (8). Gels were dried and exposed to Kodak X-Omat AR film at −80 °C for appropriate lengths of time (12 h to 3 days).

Immunoprecipitation—ADP-ribosylated Na^+ channel proteins were immunoprecipitated from a mixture of solubilized A6 membranes using the specific Na^+ channel antibody. An appropriate dilution of antiserum (1:50) or preimmune serum was incubated with solubilized membrane proteins for 2 h, then 100 μl of a 10% solution of washed Pansorbin cells (Calbiochem) were added to each tube, and the subsequent suspension was incubated at 4 °C for an additional 30 min. Subsequently, the suspension was pelleted by spinning in an Eppendorf microcentrifuge for 2 min, after which time the pellet was washed 3 times with 1 ml of PBS containing 0.05% Nonidet P-40. After the second wash, the suspension was transferred to a clean microcentrifuge tube, and after the third and final wash, was resuspended in 100 μl of sodium dodecyl sulfate buffer and subjected to electrophoresis followed by autoradiography. ADP-ribosylated rat liver Golgi membranes were solubilized in 1% Lubrol and used for immunoprecipitation with $G\alpha_{i-3}$ antiserum or Na^+ antiserum using similar procedures.

Immunoaffinity Chromatography—A Na^+ channel rabbit polyclonal antibody was covalently linked to Reacti-gel (Pierce) as described (16, 17). Briefly, 1.25 ml of Reacti-gel was washed with 100 ml of ice-cold H_20 and added to a 2-ml microcentrifuge tube. Three mg of antibody, concentrated to a volume of 650–700 μl (glycine-Tris, pH 8.5), was added to the tube and incubated at 4 °C for 24 h. The beads were washed with 20 ml of 100 mM Na_2HPO_4, pH 8.5, to remove non-bonded antibody. Coupling efficiency was monitored by measuring the antibody concentration in solution by direct protein measurement. Unreacted binding sites were blocked with ethanolamine, pH 8.5, for another 24 h at 4 °C. The beads were again washed with 20 ml of the sodium phosphate solution. After this wash, a total of 2 mg of protein (solubilized A6 homogenate) was mixed with 1 ml of bead volume and incubated with gently end-to-end shaking for 3 h. After this final incubation, the column was rinsed extensively with Na_2HPO_4 buffer. The bound protein was eluted with 0.1 M glycine (pH 2.8) and collected in 1-ml fractions. Each fraction was immediately neutralized with 1.5 M Tris-OH (pH 8.8) and its protein content measured. Fractions were pooled, concentrated on Amicon-10 microconcentrators, and the immunoaffinity protein separated by sodium dodecyl sulfate-gel electrophoresis. This procedure isolated purified epithelial Na^+ channel protein (11). After eletrophoretic separation, the Na^+ channel subunits were transferred to nitrocellulose and probed with $G\alpha_{i-3}$ antibodies.

Immunofluorescence Localization—Cultures of A6 renal epithelial cells were fixed in 4% paraformaldehyde in PBS for 1 h at 20 °C or 16 h at 4 °C. The cells were washed in several changes of PBS, permeabilized by exposure to 0.1% Triton X-100 in PBS, and then rinsed in blocking buffer (PBS with 0.5% bovine serum albumin). The cells were incubated in diluted antiserum or control serum for 2 h at 20 °C , washed in several changes of blocking buffer and then incubated with FITC-conjugated goat anti-rabbit IgG for 1 h. Cells on coverslips or on filters were mounted in PBS/glycerol with 1% *N*-propyl gallate and viewed by conventional epifluorescence or by confocal microscopy on a Zeiss microscope fitted with a Bio-Rad 600 confocal laser imaging system.

RESULTS

Effect of Amiloride and Pertussis Toxin on Na^+ Uptake into A6 Confluent Cells—The uptake of $^{22}Na^+$ was used to assess the effect of pertussis toxin on amiloride-sensitive Na^+ channel activity in confluent monolayers of A6 cells utilizing techniques previously developed in our laboratory (5). Two-minute Na^+ uptake was assessed, which is representative of influx rate (4, 5). As shown in Table I, approximately 50% of total ^{22}Na influx into A6 cells is mediated by an amiloride-sensitive pathway. Pertussis toxin was as effective as amiloride in decreasing ^{22}Na influx (501 *versus* 489 $nmol \cdot DNA^{-1} \cdot min^{-1}$). The effects of pertussis toxin and amiloride were not additive, consistent with the conclusion that in intact cells amiloride-sensitive Na^+ uptake is completely inhibitable by pertussis toxin. This conclusion is also consistent with our studies in isolated apical membrane patches, where pertussis

TABLE I

Effect of pertussis toxin on amiloride-sensitive $^{22}Na^+$ influx into confluent A6 cells

$^{22}Na^+$ influx (J_{Na+}) was measured in the presence or absence of pertussis toxin (PTX) (100 ng/ml) and/or amiloride. No statistical difference was observed between amiloride or pertussis toxin alone *versus* amiloride plus pertussis toxin.

	J_{Na+}	
	Minus PTX	Plus PTX
	nmol·mg DNA^{-1}·min^{-1}	
Control	889 ± 190	501 ± 55[a]
Amiloride (10^{-5} M)	489 ± 59[a]	489 ± 52[a]

[a] $p < 0.025$ *versus* control for $n = 30$ filters.

toxin completely inhibits amiloride-sensitive single Na^+ channel conductance (6). These studies in intact cells (Table I) and isolated patches (6) raise the possibility that a G_i regulatory protein is intimately related to the apical membrane Na^+ channel in A6 cells. The experiments described below were designed to address this question.

Localization of Na^+ Channel and α_{i-3} Subunit in Adjacent Apical Domains of A6 Cells—Conventional epifluorescence and confocal imaging were used to localize the Na^+ channel on apical microvilli of the A6 cells in a similar distribution to that previously reported (18). Confocal imaging further showed that the Na^+ channel staining was specifically limited to the protruding portions of the typical finger- or ridge-like microvilli on the apical cell surface (Fig. 1*A*) and there was no staining on the basal or lateral cell surfaces (data not shown). Immunofluorescence of A6 cells with an antibody specific for the α_{i-3} subunit showed punctate staining of this protein throughout the cytoplasm and on membranes of the perinuclear Golgi complex, similar to the localization seen previously in LLC-PK_1 renal epithelial cells and NRK cells (7, 8). In contrast to LLC-PK_1 and NRK cells, where it was most concentrated on Golgi membranes, α_{i-3} was found to be most abundant at the apical pole of the A6 cells. Confocal imaging and comparison of pixel intensities at different levels through the A6 cell layer revealed that α_{i-3} is most heavily concentrated in a 3-μm layer associated with the apical cell membrane, just at the base of the microvilli, although the staining did not extend into the projections themselves (Fig. 1, *B* and *C*). This localization confirms that the α_{i-3} is present at the apical membrane of the A6 cells in a distinct, but adjacent, domain to the Na^+ channel.

ADP-ribosylation of Solubilized Membrane Proteins from A6 Cells—ADP-ribosylation of solubilized A6 membrane proteins followed by electrophoresis demonstrated three major protein bands at 95, 66, and 41 kDa, respectively (Fig. 2, *lane 1*). The 66-kDa band is a frequent contaminant of such studies reflecting, at least in part, serum albumin used in toxin activation.[2] Following immunoprecipitation with the Na^+ channel antibody and electrophoresis, two ADP-ribosylated bands were seen: one at 95 kDa and the other at 41 kDa. Neither protein was immunoprecipitated with preimmune serum (data not shown).

Since amiloride is known to bind to at least one member of the Na^+ channel complex (*e.g.* 130/150-kDa protein) (10) it was of interest to see if amiloride binding altered ADP-ribosylation of any of the Na^+ channel proteins. As seen in Fig. 3, the presence of increasing amounts of amiloride, which functionally block Na^+ channel activity, had no effect on the ADP-ribosylation of either the 95- or 41-kDa proteins.

Co-purification of α_{i-3} in the Na^+ Channel Complex—The HPLC-purified Na^+ channel complex containing a number of proteins (Fig. 4*A*) was immunoblotted with a polyclonal Na^+ channel antibody and with preimmune serum as a control (Fig. 4*B*). As previously described, polyclonal Na^+ channel antibodies variably label only some of the proteins in the complex; this antiserum recognized proteins at 320, 150, and 95 kDa (Fig. 4*B*, *lane 2*). Transfers of the purified Na^+ channel complex were probed with the $G\alpha_{i-3}$ antibody (Fig. 4*C*, *lane 4*), which showed that a 41-kDa protein, corresponding to $G\alpha_{i-3}$, was part of this purified complex. In order to further demonstrate that α_{i-3} is actually part of the Na^+ channel complex, this polyclonal Na^+ channel antibody was used to construct an immunoaffinity column for purification of Na^+ channel complexes from detergent-solubilized A6 cell homogenates. Affinity-bound proteins, eluted from the Na^+ channel column with low pH buffer, were electrophoresed and transferred for Western blotting with the $G\alpha_{i-3}$ antibody (Fig. 4*C*). The 41-kDa, α_{i-3} protein was identified in the eluate from the affinity column (Fig. 4*C*, *lane 6*), while there was no protein detected with the control, nonimmune serum (Fig. 4*C*, *lane 5*). These results suggest that $G\alpha_{i-3}$ is co-purified as part of a multi-protein Na^+ channel complex.

In order to confirm that $G\alpha_{i-3}$ itself is not recognized by the Na^+ channel antibody, this antibody was used to Western blot an enriched source of $G\alpha_{i-3}$. $G\alpha_{i-3}$ has previously been localized on Golgi membranes (7, 8). When Golgi membranes isolated from rat liver were used for Western blotting, the antibody to $G\alpha_{i-3}$ recognized a 41-kDa protein (Fig. 5*A*, *lane 1*), but there was no staining of this band, or any other protein band on Golgi membranes, with the Na^+ channel antibody (Fig. 5*A*, *lane 2*). In addition, we confirmed that the Na^+ channel antibody cannot immunoprecipitate $G\alpha_{i-3}$ from the Golgi membranes in the absence of other Na^+ channel proteins. Golgi membranes were ADP-ribosylated with pertussis toxin to label the α_{i-3} subunit (Fig. 5*B*, *lane 3*), and then solubilized Golgi membrane proteins were used for immunoprecipitation with Na^+ channel or $G\alpha_{i-3}$ antibodies. The $G\alpha_{i-3}$ antibody precipitated some of the ADP-ribosylated α_{i-3} subunit (Fig. 5*B*, *lane 4*), but the Na^+ channel antibody was not able to immunoprecipitate the α_{i-3} subunit (Fig. 5*B*, *lane 5*), providing further evidence that the Na^+ channel antibody does not directly recognize $G\alpha_{i-3}$.

Specificity of α_{i-3} as a Functional Stimulator of Na^+ Channel Activity—The apical membrane localization and co-immunoprecipitation of the α_{i-3} subunit with the Na^+ channel suggested a specific functional role for α_{i-3} in Na^+ channel regulation. Such a role was confirmed by the data presented in Table II. In the presence of pertussis toxin for 1 min there is a decrease in the percent open time of spontaneous Na^+ channels, which is rapidly reversed by the addition of 20 pM α_{i-3}, as we have previously shown (6). In contrast, the addition of a homologous α_i subunit, α_{i-2}, had no effect on the recovery of Na^+ channel activity in the presence of pertussis toxin. As can be seen in Table II, Na^+ channel activity continued to decay in the presence of pertussis toxin and α_{i-2} over 5 min, consistent with our previous data showing that Na^+ channel activity is near zero after 3 min of pertussis toxin exposure (6). The addition of another homologous $G\alpha_i$ subunit, α_{i-1}, also did not alter single Na^+ channel conductance in the presence of pertussis toxin (data not shown).

DISCUSSION

The transepithelial transport of Na^+ is a major function of renal epithelial cells. Renal Na^+ channel activity is known to be under hormonal control (such as vasopressin or aldosterone), and classical second messenger signaling pathways in-

[2] D. A. Ausiello, J. L. Stow, H. F. Cantiello, J. B. de Almeida, and D. J. Benos, unpublished observations.

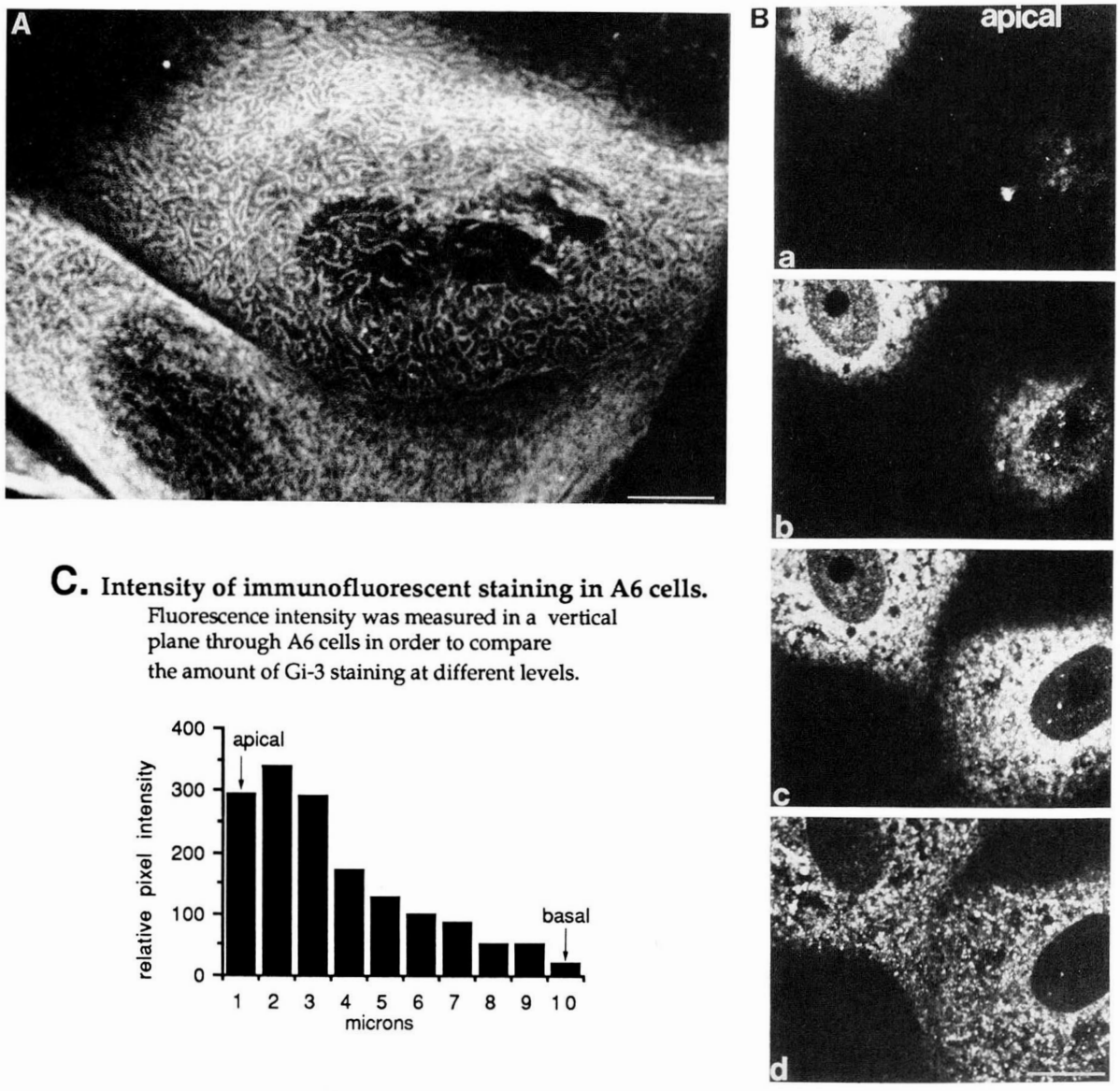

FIG. 1. **Na^+ channel is localized on the apical plasma membrane of A6 cells.** *A*, immunofluorescence localization of the Na^+ channel on the apical membrane of A6 cells. Cells were fixed and permeabilized as described under "Experimental Procedures" and reacted with a specific polyclonal antibody to the purified Na^+ channel, followed by an FITC-second antibody. Cells were viewed in a Zeiss photomicroscope fitted with a Bio-Rad confocal imaging system. Images were collected at 1-μm intervals in a Z series (vertical axis) through the cell layer. The image shown here was taken at the level of the apical cell membrane and shows staining of the Na^+ channel over the entire microvillar surface of the cell. The Na^+ channel staining is restricted to the protruding microvilli and is not present on other domains of the cell membrane. *Bar*, 10 μm. *B* and *C*, $G\alpha_{i-3}$ is concentrated near the apical membrane of A6 cells. Immunofluorescence staining was used to localize the $G_{\alpha i-3}$ subunit in A6 cells. The A6 cells were fixed and permeabilized as described and incubated with a specific α_{i-3} peptide antibody followed by FITC-goat anti-rabbit IgG. Cells were viewed in a Zeiss photomicroscope and imaged on a Bio-Rad confocal imaging system. Ten images were collected at 1-μm intervals in a Z series (vertical axis) through the cells from the apical to the basal level. This figure shows representative images from the apical section (*panel a*) down to the middle of the cell (*panel d*). Punctate staining of the α_{i-3} in these cells is found throughout the cytoplasm. There is a concentration of larger vesicular structures in the perinuclear area (seen in *panel d*) which corresponds to Golgi staining. There is a significantly greater intensity of α_{i-3} staining in apical sections (*panels a* and *b*) than in sections towards the middle (*panels c* and *d*) or base of the cell (not shown). The apical staining in *panels a* and *b* corresponds to a level at the base of the microvilli in each of the two cells pictured which are at different heights. *C*, bar, 10 μm. The fluorescence intensity was measured along a line through the cell layer in order to quantitate the amount of α_{i-3} staining at different levels through the cell. This confirmed that the staining of α_{i-3} is most intense in a 3-μm band at the apical pole of the cell and is greatest at the level of the base of the microvilli.

volving protein kinase A or protein kinase C have been shown to activate or inhibit the amiloride-sensitive Na^+ channel, respectively, in A6 cells and other renal epithelia (1). However, how these signals are responsible for Na^+ channel activity at the apical membrane is not known. Recently, utilizing the patch-clamp technique, we have defined the modulation of Na^+ channel activity in A6 cells by a pathway(s) involving endogenous pertussis toxin-sensitive G_i proteins (6) and phospholipids (19) located at the apical membrane which are topologically separated from second messenger pathways gen-

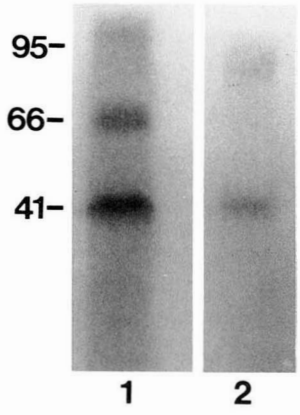

FIG. 2. **Pertussis toxin induced ADP-ribosylation of Na^+ channel complex.** Autoradiograph of pertussis toxin induced ADP-ribosylation of solubilized A6 membranes (30 μg of protein) (*lane 1*) and proteins immunoprecipitated from solubilized membranes with Na^+ channel antibody (*lane 2*). Three major protein bands are ribosylated in these membranes; the apparent molecular weights of these proteins are marked according to the molecular weight standards. Only two of these proteins, a 95 and a 41-kDa protein, are precipitated by the Na^+ channel antibody (*lane 2*).

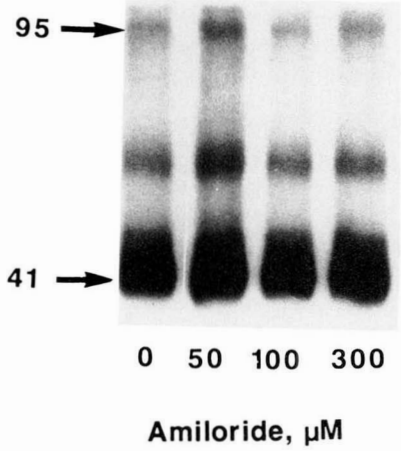

FIG. 3. **Effect of amiloride on ADP-ribosylation.** Autoradiograph of pertussis toxin induced ^{32}P ADP-ribosylation of solubilized A6 membrane proteins (30 μg of protein/lane) in the presence of increasing concentrations (0–300 μM) of amiloride. The presence of amiloride has no effect on the level of ribosylation of the labeled proteins.

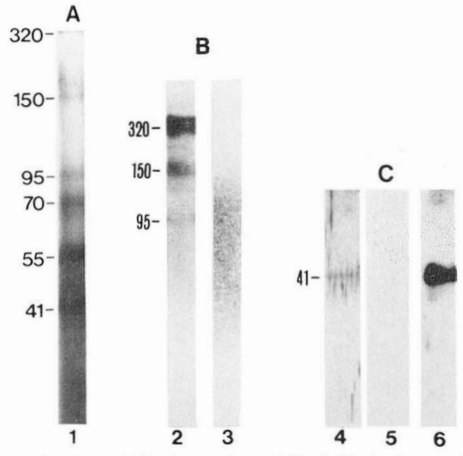

FIG. 4. **Western blotting of purified Na^+ channel complex.** *A, HPLC-purified Na^+* channel complex from solubilized A6 membranes was electrophoresed on 8% polyacrylamide gels and silver-stained to show the proteins comprising the complex (320, 150, 95, 70, 55, and 41 kDa) (*lane 1*). *B*, purified Na^+ channel complex proteins (200 ng/lane) were loaded onto polyacrylamide gels, electrophoresed, and then transferred to nitrocellulose for Western blotting. Transfers were probed either with preimmune serum (*lane 3*) or with the Na^+ channel antibody (*lane 2*) followed by alkaline phosphatase conjugates. This Na^+ channel antibody typically recognizes the 320-, 150-, and 95-kDa bands of the complex by Western blotting (*lane 2*), although it immunoprecipitates the whole complex (10). *C*, the HPLC-purified Na^+ channel complex (200 ng/lane) was Western blotted with the $G\alpha_{i\text{-}3}$ antibody followed by an alkaline phosphatase conjugate (*lane 4*). The 41-kDa band of $\alpha_{i\text{-}3}$ was detected in the Na^+ channel complex. Immunoaffinity column-purified Na^+ channel complex (200 ng/lane) (*lanes 5* and *6*) was Western blotted with anti-$\alpha_{i\text{-}3}$ serum (*lane 6*) or without serum as a control (*lane 5*), followed by detection with ^{125}I-labeled protein A. There was no specific binding of ^{125}I-protein A in the absence of antiserum (*lane 5*); the $\alpha_{i\text{-}3}$ antibody labeled a single band at 41 kDa (*lane 6*) indicating that $\alpha_{i\text{-}3}$ is present in the affinity-purified Na^+ channel complex.

erated at the basolateral membrane. Since the exogenous $\alpha_{i\text{-}3}$ subunit of G_i was capable of substituting for the endogenous G_i protein in modulating Na^+ channel activity (6, 19), it seemed probable that $\alpha_{i\text{-}3}$ was a regulatory protein associated with the Na^+ channel. Therefore the present study was designed to demonstrate the apical membrane localization and co-purification of the $\alpha_{i\text{-}3}$ subunit along with the six major proteins that make up the Na^+ channel complex in the apical membrane. We have previously shown that the ~300-kDa protein of this complex is a substrate for protein kinase A phosphorylation, although its function is as yet unknown (20). The 130–150-kDa protein binds amiloride, although it is not known if this subunit itself confers the conductive properties involved in Na^+ channel activity (10). The other proteins in the Na^+ channel complex are as yet undefined, although it is reasonable to speculate that they may be regulatory proteins forming a complex with the Na^+ channel itself.

In the present study, immunocytochemistry, utilizing specific antibodies, localized the $\alpha_{i\text{-}3}$ subunit to a subapical membrane domain at the level of the base of the microvilli in A6 cells. In recent studies we have shown that $G\alpha_{i\text{-}3}$ is most heavily concentrated on the Golgi membranes of other epithelial cells including LLC-PK_1 and NRK cells (7, 8) where it functions as a regulator of the secretory pathway. Consistent with this general finding, there is also $G\alpha_{i\text{-}3}$ staining on perinuclear Golgi membranes in A6 cells. However, the most intense staining of $G\alpha_{i\text{-}3}$ in the A6 cells is at the apical cell membrane, showing that in some cells, such as A6, $G\alpha_{i\text{-}3}$ is localized in more than one domain. This suggests that in certain cells $G\alpha_{i\text{-}3}$ may perform multiple functions. The subapical membrane domain containing $G\alpha_{i\text{-}3}$ in A6 cells is distinct from, but closely adjacent to, the Na^+ channel-rich domains on the apical microvilli. Although this localization shows heavy concentrations of $\alpha_{i\text{-}3}$ in the sub-apical domain, we cannot rule out the possibility that undetectable amounts of $\alpha_{i\text{-}3}$ in the microvilli themselves are responsible for regulating the Na^+ channel. However, the localization of Na^+ channels and $G\alpha_{i\text{-}3}$ in adjacent domains could suggest that G_i protein coupling to the Na^+ channel may not be direct, but rather its stimulatory capacity is generated by further intermediary signal transducing pathways. These data are consistent with our recent observation that G_i protein modulation of Na^+ channel activity is the result of activation of phospholipase and lipoxygenase pathways generating phospholipids which are responsible for Na^+ channel activity (19). Of interest is our recent observation that actin filament network organization is also a regulator of the Na^+ channel (21). Taken together these data would support the conclusion that an ion channel regulatory complex consisting of a G_i protein, phospholipase, lipoxygenase, and actin-binding proteins could be

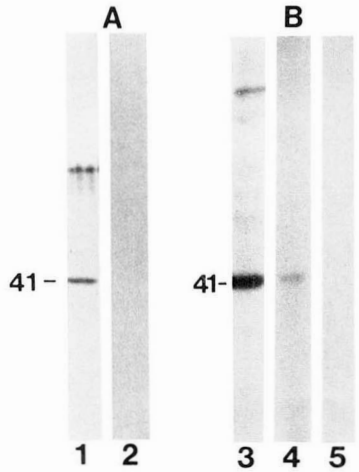

FIG. 5. **Western blotting of isolated rat liver Golgi membranes.** *A*, isolated Golgi membranes from rat liver, used as an enriched source of Gα_{i-3}, were electrophoresed on gels (50 μg/lane), transferred, and Western blotted with antibodies specific for α_{i-3} (*lane 1*) and Na^+ channel (*lane 2*). Reaction of the Golgi membranes with anti-α_{i-3} serum confirmed the presence of the 41-kDa α_{i-3} subunit (*lane 1*). There is nonspecific binding of antibody to albumin at a higher molecular weight. Reaction of the Golgi membranes with the Na^+ channel antibody gave no staining of the 41-kDa band or of any other proteins (*lane 2*) showing that the Na^+ channel antibody does not recognize the α_{i-3} protein. *B*, isolated Golgi membranes were ADP-ribosylated with pertussis toxin. The major ADP-ribosylated protein in these membranes is the 41-kDa, Gα_{i-3} (*lane 3*). Solubilized membranes were then used for immunoprecipitation with the Gα_{i-3} antiserum or with Na^+ channel antiserum. The α_{i-3} antibody precipitated the 41-kDa α_{i-3} subunit (*lane 4*), but the Na^+ channel antibody did not precipitate the labeled Gα_{i-3} (*lane 5*).

TABLE II

Effect of purified α_{i-2} or α_{i-3} subunits on amiloride-sensitive Na^+ channel activity as measured by the patch-clamp technique

Pertussis toxin (PTX) (100 ng/ml) was added for 1 min, following which % open time and mean conductance, γ, were determined (PTX columns). At 1 min, α_{i-3} or α_{i-2} was added and the same parameters were again determined at 5 min.

	Control	PTX	α_{i-3} (20 pM)
Open time (%)	67.5 ± 6.2	16.4 ± 7.0[a]	55.7 ± 9.8
γ(pS)[b]	9.6 ± 2.1	9.7 ± 2.1	12.1 ± 4.4
	Control	PTX	α_{i-2} (20 pM)
Open time (%)	69.1 ± 10.3	19.0 ± 15.5[a]	8.1 ± 2.5[a]
γ(pS)	10.6 ± 2.1	8.6 ± 2.8	8.2 ± 2.5

[a] $p < 0.02$ *versus* control, $n = 3$.
[b] pS, picosiemens.

co-associated in the apical membrane of the A6 cells. Recently, Smith *et al.* (22) have demonstrated that actin and the actin-binding proteins ankyrin and fodrin, which link actin filaments with the plasma membrane, are able to bind to proteins in the Na^+ channel complex from renal epithelia. It is therefore possible that the purified 700-kDa complex is made up of a number of modulatory proteins sequestered through non-covalent linkages with cytoskeletal proteins, such as ankyrin. Further studies will be necessary to prove this hypothesis.

Pertussis toxin-induced ADP-ribosylation of the Na^+ channel complex has yielded data supporting two conclusions. The first is that since the 41-kDa, α_{i-3} protein is a substrate for ADP-ribosylation by pertussis toxin, the βγ subunit should also be present . Although we do not have direct evidence to support this conclusion, to date no heterotrimeric Gα_i subunits have been ADP-ribosylated by pertussis toxin in the absence of the β (and probably γ) subunit (23). The second conclusion is that the inability of amiloride to alter the pertussis toxin-mediated ribosylation of both the 95- and 41-kDa proteins suggest that there is not a direct interaction between these two proteins and the 130/150-kDa amiloride-binding protein. This conclusion is supported by the observations that G proteins binding to receptors or effectors have a known hysteresis, such that the modulation of receptor or effector has a direct effect on the G protein (24). This observation is in contrast to that reported by Anand-Srivastava (25), who found that the pertussis toxin-catalyzed ADP-ribosylation of purified Gα_i from bovine brain was prevented by amiloride. Although the data in the present report are indirect and subject to alternative explanations, it seems likely that if the amiloride-bound Na^+ channel component of the Na^+ complex (presumably the 150-kDa protein) were structurally altered in a way to facilitate G protein subunit dissociation or reassociation, one might have expected an alteration in the pertussis toxin ADP-ribosylation pattern. This did not occur. It is therefore reasonable to conclude that the presence or absence of the amiloride-binding protein does not alter the degree of dissociation of the $\alpha_{i-3}/\beta\gamma$ subunit in the apical membrane. It remains to be tested, however, whether alterations in G_{i-3} activity, either by guanine nucleotides or pertussis toxin (which is known to disrupt the interaction of G proteins with receptors and probably effectors (23, 24)) have an effect on amiloride binding to the purported Na^+ channel protein. Finally, it is of interest that an additional protein, a 95-kDa protein, is also ADP-ribosylated in the Na^+ channel complex. To our knowledge there are no previously identified pertussis toxin-sensitive G proteins of this apparent molecular weight. It does not appear likely that this effect could be explained by an oligomerization of known G proteins of smaller molecular weights since none of the G protein antibodies (data not shown) immunoblotted this protein on Western blots. Therefore, no further conclusions can be reached concerning the functional significance of this pertussis toxin-sensitive 95-kDa protein.

In conclusion, we have demonstrated that the regulation of amiloride-sensitive Na^+ channels in the apical membrane of A6 cells by pertussis toxin and guanine nucleotides is consistent with the role of an endogenous α_{i-3}-like protein localized in adjacent apical microdomains with the Na^+ channel. This localization is also consistent with data that a novel signal transduction pathway is present in these apical membranes, consisting of sequential activation of G proteins, phospholipases, lipoxygenases, and the Na^+ channel (19), possibly coupled through the actin cytoskeleton (21). It is possible that the other proteins co-purified as the Na^+ channel complex might include members of this pathway providing for a functional assembly of regulatory proteins coordinately capable of regulating Na^+ channel activity.

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