Forskolin-induced Cell Shrinkage and Apical Translocation of Functional Enhanced Green Fluorescent Protein-Human αENaC in H441 Lung Epithelial Cell Monolayers*

Alison M. Woollhead and Deborah L. Baines

From the Division of Basic Medical Sciences, Ion Channels and Cell Signaling Centre, St. Georges’ University of London, Cranmer Terrace, Tooting, London SW17 0RE, United Kingdom

Elevation of intracellular cAMP increases fluid re-absorption in the lung by raising amiloride-sensitive Na⁺ transport through the apically localized epithelial, amiloride-sensitive Na⁺ channel (ENaC). However, the signaling pathways mediating this response are still not fully understood. We show that inhibition of protein-tyrosine kinase (PTK) with Genistein and protein kinase A (PKA) with KT5720, decreased forskolin-stimulated amiloride-sensitive short circuit current (Iₛₒ) across H441 adult human lung epithelial cell monolayers. KT5720 also decreased basal Iₛₒ. Stable expression of green fluorescent protein (GFP)-labeled human αENaC in H441 cells was used to investigate dynamic changes in the cellular localization of this protein in response to forskolin. Reverse transcription-PCR and immunoblotting analysis revealed two clones expressing a truncated (αC3-5) and full-length (αC3-3) EGFP-hαENaC protein. Only the αC3-3 clone displayed dome formation and exhibited a 50% increase in basal and forskolin-stimulated amiloride-sensitive Iₛₒ indicating that the full-length protein was required for functional activity. Apical surface biotinylation and real-time confocal microscopy demonstrated that EGFP-hαENaC (αC3-3) translocated to the apical membrane in response to forskolin in a Brefeldin A-sensitive manner. This effect was completely inhibited by Genistein but only partially inhibited by KT5720. Forskolin also induced a reduction in the height of cells within αC3-3 monolayers, indicative of cell shrinkage. This effect was inhibited by KT5720 but not by Genistein or Brefeldin A. These data show that forskolin activates PKA-sensitive cell shrinkage in adult human H441 lung epithelial cell monolayers, which induces a PTK-sensitive translocation of EGFP-hαENaC subunits to the apical membrane and increases amiloride-sensitive Na⁺ transport.

Transport of Na⁺ through the amiloride-sensitive Na⁺ channel (ENaC), found in the apical membrane of polarized lung epithelial cells, is considered the rate-limiting step for transepithelial Na⁺ movement and the regulation of lung fluid re-absorption via osmosis (1). ENaC comprises three subunits α, β, and γ (2). It has been shown that the α-subunit is capable of forming an Na⁺-conducting pore and that β and γ augment its conductance (2). Channels may be formed when αENaC is expressed independently of the β and γ subunits (3, 4). For this reason, expression of the α-subunit is considered of critical importance. This has been demonstrated in αENaC knock-out mice where death of newborn mice lacking αENaC was shown to be the result of an inability to clear their lungs of fluid (5). A more recent study has extended these findings and indicated that the low mRNA abundance level of αENaC in nasal epithelium of premature infants is associated with respiratory failure (6).

At birth, the lungs undergo a transition from a fluid-filled to that of an air-filled (post-natal) state, a requirement for normal breathing and efficient gas-exchange. In the fetal sheep and guinea pig lung, a surge in plasma adrenaline around and during the time of birth has been shown to correlate with increased amiloride-sensitive fluid re-absorption (7, 8). The ability of adrenaline to mediate lung fluid re-absorption has also been shown to retain throughout adult life (9). Adrenaline is thought to act through the cAMP second messenger system, and its effects on fluid absorption can be mimicked by agents that increase intracellular cAMP such as forskolin (10–12). In the rat fetal distal lung cell, the action of cAMP is thought to increase the recruitment of ENaC subunits to the apical membrane by increased trafficking via a Brefeldin A-sensitive pathway (13). This phenomenon is also upheld in adult human bronchiolar epithelial H441 cells (14) where the apical conductance of ENaC is also increased by cAMP (14–16). However, which ENaC subunits are recruited to the apical membrane and the signaling pathways involved remains unclear in lung cells. Elevation of cAMP by β₂-adrenoreceptor agonists is classically associated with activation of protein kinase A (PKA). However, evidence indicates that the effect of β₂-adrenoreceptor activation on the trafficking of ENaC may not involve a direct effect of PKA and may utilize alternative pathways that involve protein-tyrosine kinase (PTK) (17, 18). The dissection of the mechanisms by which elevated cAMP increases ENaC subunit trafficking in the lung cell membrane is therefore the key to our understanding of how this channel contributes to the regulation of lung fluid homeostasis.

In A6 renal epithelial cells, it has been shown by confocal microscopy that sub-membrane pools of ENaC protein exist that can be translocated to the membrane when stimulated with forskolin or insulin (19, 20). This indicates that active trafficking of ENaC subunit proteins to the membrane is an important regulatory process. Evidence also indicates that the trafficking of ENaC subunits to the apical membrane can also take place in a non-coordinate manner (21).

In this study, we have used a combination of complementary approaches to investigate the role of PKA and PTK in mediating the effects of forskolin-elevated cAMP on amiloride-sensitive Na⁺ transport in human lung epithelial cell monolayers. We have generated green fluorescent protein (GFP)-labeled human αENaC chimeric proteins and stably expressed them in H441 lung epithelial cells. Using measurement of short-circuit current, apical surface biotinylation, immunoblota-

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1 To whom correspondence should be addressed. Tel.: 44-0208-725-0916; Fax: 44-0208-725-2993; E-mail: d.baines@sgul.ac.uk.

2 The abbreviations used are: ENaC, amiloride-sensitive epithelial Na⁺ channel; PKA, protein kinase A; PTK, protein-tyrosine kinase; GFP, green fluorescent protein; RT, reverse transcription; IBMX, isobutylmethylxanthine; WGA, wheat germ agglutinin; FDLE, fetal distal lung epithelial.
ting, and real-time confocal microscopy, we have investigated the relationship between dynamic changes in EGFP-hαENaC distribution and functional Na\(^+\) transport in lung cells in response to forskolin stimulation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**H441 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 medium supplemented with fetal bovine serum (10%) (Immune Systems Ltd.), L-glutamine (2 mM), sodium pyruvate (1 mM), insulin (5 μg/ml), transferrin (5 μg/ml), sodium selenite (10 nM), and antibiotics (penicillin/streptomycin). Cells were seeded in 25-cm\(^2\) flasks and incubated in a humidified atmosphere of 5% CO\(_2\)–95% O\(_2\) at 37 °C.

**Design of EGFP-human αENaC Construct—**cDNA encoding human (h) αENaC was first subcloned into pGEMT Easy (Promega) using standard procedures. The expression vector EGFP-hαENaC (paC3) was generated by amplifying the coding region of hαENaC using primers that incorporated the restriction enzyme sites XhoI/BamHI (sense, 5’-agagactctgacaggaatgagenccagttgga3’; antisense, 5’-tcgacatggacatctgagacagtcc-3’). PCR products were then cloned directionally and in-frame into the XhoI/BamHI sites of pEGFP-C3 (Clontech). Plasmid sequences and reading frame were confirmed by DNA sequencing (Sequence Laboratories London Ltd.). Primers were also designed within regions of both the EGFP and hαENaC sequence for sequence analysis and RT-PCR amplification of EGFP-hαENαC mRNA sequences in transfected cells. From 5’-3’ these were β\(_{5}\), atggtcctgctggagttcgt; ASH, acccgatgtatgagagagctcgagggaattatggaggggaacaagctgga3’; antisense, 5’-tgtgtgtggtccttataagccgccccagttgga-3’.

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**RT-PCR—**Total RNA was extracted from control and transfected H441 cells using Tri-Reagent (Sigma). cDNA was synthesized from 2 μg of RNA using 1 unit of avian myeloblastosis virus-reverse transcriptase (Promega) and 100 nM oligo-dT\(_{15}\) primers. RT-PCR was performed using 20-bp primer sequences derived from regions within the pEGFP C3 vector and hαENαC coding regions in a PCR reaction mix (75 mM Tris, pH 9.0, 20 mM (NH\(_4\))\(_2\)SO\(_4\), 1.5 mM MgCl\(_2\), 1% Tween 20, 250 nM of each primer, and 0.2 unit of AGS gold polymerase (Hybaid, Ashford, Middlesex, UK) and a cycling protocol of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min for 40 cycles. PCR products were fractionated on 1.2% agarose gels and visualized using ethidium bromide staining and UV fluorescence.

**Surface Biotinylation—**Control H441 cells or those stably expressing full-length EGFP-hαENαC (αC3-3) were seeded onto 24-mm Transwells (Costar Transwells, Corning) and cultured overnight. The following day, the serum in the medium was replaced with 4% charcoal-stripped serum, and the supplementation also included thymoxine (10 nM) and dexamethasone (200 nM). Cells were cultured for 6 days at air interface, and Transwells supporting resistive monolayers of cells (>500 Ω-cm\(^{-2}\)) were treated bilaterally with 10 μM forskolin (activator of adenyl cyclase) and 100 μM 3-isobutyl-1-methylxanthine (IBMX, phosphodiesterase inhibitor) for 30 min at 37 °C, or treated with vehicle only as a control. Forskolin and IBMX are both known to elevate cAMP levels (22); therefore, stimulation with these agents is referred to as "forskolin stimulation" throughout this report. Transwells were washed with ice-cold physiological salt solution (in mM): NaCl, 117; NaHCO\(_3\), 25; KCl, 4.7; MgSO\(_4\), 1.2; KH\(_2\)PO\(_4\), 1.2; CaCl\(_2\), 2.5 (equilibrated with 5% CO\(_2\) to pH 7.3–7.4) then the apical membrane was biotinylated using 0.5 mg/ml S-S-biotin (Pierce) in borate buffer (in mM) (NaCl, 85; KCl, 4; Na\(_2\)B\(_4\)O\(_7\), 15), pH 9, for 20 min (23). The reaction was stopped by adding a double volume of fetal bovine serum-containing medium on the apical surface. Monolayers were then washed with ice-cold physiological salt solution, and cells were scraped into biotinylation lysis buffer (0.4% deoxycholate, 1% Triton X-100, 50 mM EGTA, 10 mM Tris-Cl, pH 7.4) containing 1% protease inhibitors (Sigma) and incubated at room temperature for 10 min. Non-solubilized protein was removed by centrifugation, and protein concentrations of the supernatants were determined (Bio-Rad). Similar concentrations of protein from treated and untreated Transwells (~1 mg) were incubated overnight at 4 °C with 30 μl of immobilized streptavidin beads (Pierce). The following day, the beads were washed extensively in biotinylation lysis buffer, and protein was eluted in sample buffer containing reducing agent (Invitrogen, according to the manufacturer’s instructions). Samples were incubated for 30 min at room temperature, heated at 95 °C for 5 min, fractionated on SDS-PAGE gels, and immunoblotted with anti-αENαC (Abcam, UK). Immunostained proteins were visualized using ECL (Amersham Biosciences).

**Immunoprecipitation—**H441 cells and clones were washed briefly with ice-cold phosphate-buffered saline and harvested into ice-cold tissue lysis buffer (in mM): Tris, 50 (pH 7.4); NaCl, 150; NaF, 50; sodium pyrophosphate, 5; EDTA, 1; EGTA, 1; dithiothreitol, 1; 1% v/v Triton X-100; and 1% protease inhibitor mixture (Sigma). Lysates were pre-cleared with 3% Protein A-Sepharose (Sigma) overnight at 4 °C on a rocker/roller. Pre-cleared supernatants were then incubated with anti-EGFP (Abcam) for 1 h on ice. Protein-antibody complexes were bound to a fresh column of beads for a further hour. After several washes in lysis buffer, proteins bound to the beads were eluted into sample buffer (as above) heated at 95 °C for 5 min, subjected to Western blot analysis, immunostained with anti-αENαC (Abcam), and visualized using ECL (Amersham Biosciences).

**Functional Experiments—**Confluent flasks of H441 cell clones were seeded at 1:12 confluent density on Snapwell clear membranes (Costar Transwells, Corning) and treated as described for Transwells (see above). Because basal levels of ion transport across monolayers vary considerably between batches of cells, all experiments (treatments or otherwise) were carried out on cells that were plated on the same day, and results are compiled from at least three sets of paired data, analyzed in parallel. Monolayers, were pre-treated in culture (bilaterally) for 30 min with PKA inhibitor KT5720 (1 μM), PTK inhibitor Genistein (100 μM), or vehicle control. Snapwells supporting resistive monolayers of H441 cells (>500 Ω-cm\(^{-2}\)) were mounted in Ussing chambers, and I\(_{sc}\) was measured as previously described (24). Forskolin-stimulated Na\(^+\) transport was measured in H441 cell clones before and after pre-treatment with inhibitors (see above). 10 μM forskolin and 100 μM IBMX were added to the solution perfusing both the apical and basolateral side of the monolayer. Changes in I\(_{sc}\) were monitored for 15–20 min until a new stable level had been reached. At this point, 10 μM amiloride (EC\(_{50}\),...
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In the live cell studies, WGA staining was not sufficient to provide optimal intensity and did not enable us to show high quality double stained images. These studies were conducted at 37 °C, which may have affected the association/dissociation of the WGA with its substrate. Similar problems with WGA have previously been reported in kidney epithelial cells (19). However, fluorescence was sufficient to provide us with a means of localizing apical z-planes that contained both WGA and EGFP fluorescence. Furthermore, because EGFP-hαENaC is distributed throughout the cell, the entire cell could be visualized. The semi-permeable filter of the Snapwells also had an innate fluorescence allowing us to definitively orientate the cells. Sequential z-sections were acquired at 1-μm intervals from this point encompassing the entire monolayer. A time-series over a period of 40 min was acquired automatically at 5-min intervals. The time course for these experiments was increased compared with the functional studies as they were carried out at room temperature not 37 °C. Any microscope drift was compensated for by using the innate fluorescence of the filter as a reference point by which to align the image stacks. After the first z-series was acquired, 10 μM forskolin and 100 μM IBMX were added to the media bathing the cells, and sequential z-sections were acquired for a further 40 min. Cell movement through an optical plane was initially a confounding issue as the cells exhibited dynamic changes (shrinkage) during treatment. However, using the criteria outlined above we were able to track the movement through an optical plane. Images representing the apical surface of the cell in the x/y-plane were used to compile fluorescence intensity measurements from a number of independent cells (n = 11) within a monolayer. These included cells with both high and low levels of expression of the ENaC or EGFP alone.

Results are shown as mean ± S.E.

0.7 μM)³ was added to the apical solution, and amiloride-sensitive Iₘ was calculated.

Microscopy—Confluent monolayers of αC3–3 cells, grown on plastic, formed fluid-filled hemicylindrical domes, which were imaged using an inverted Nikon Eclipse TE300 light microscope with a ×10 objective. For confocal microscopy, cells were either plated on coverslips (no. 1, BDH, UK) or grown on Snapwell clear membranes as described above. Cells grown on coverslips were treated in culture with 10 μM forskolin and 100 μM IBMX or vehicle for 30 min, washed in ice-cold phosphate buffered saline, and then incubated on ice with rhodamine-conjugated wheat germ agglutinin (WGA, 2 μg/ml, Molecular Probes) for 30 min prior to fixation with 1% paraformaldehyde (Sigma). Coverslips were mounted in fluorescent mounting medium (Mowiol, Calbiochem) ready to be imaged the following day. In the live cell studies, Snapwells supporting resistive monolayers of clones were either treated in culture with KT5720 (1 μM), Genistein (100 μM), or the vesicle trafficking inhibitor, Brefeldin A (1 μM) or left untreated as control. Snapwell inserts were then placed on coverslips (no. 1, Nunc) secured on the stage-plate and filled with 500 μl of phenol-red-free culture media (Invitrogen) to bathe the apical side of the monolayer. Confocal microscopy was performed with an inverted Zeiss LSM 510 Meta microscope, equipped with an oil immersion objective (×40), which was used for all imaging. For the dual imaging of EGFP and WGA, fluorescent images were collected in the multichannel mode by exciting the fluorophores at 488 and 568 nm, respectively. The depth of the acquired z-series was defined by the EGFP-associated fluorescence within the cell. WGA, which was used to specifically label cell membranes, provided us with a means to identify z-planes in which the labeled surface proteins co-localized with EGFP-tagged proteins within the apical membrane. In fixed cells differential and heterogeneous staining was observed in both control H441 cells and those expressing EGFP-hαENaC or EGFP alone.

³D. Baines, unpublished data.

In Figure 1, characterization of forskolin-stimulated Iₑ across H441 cell monolayers. In A: panel i, transepithelial short circuit current (Iₑ) was measured before (control) and after treatment with 10 μM forskolin/100 μM IBMX (F/I) followed by apical application of 10 μM amiloride (amiloride). Panel ii, transepithelial short circuit current (Iₑ) was measured before (control) and after apical application of 10 μM amiloride (amiloride) followed by treatment with 10 μM forskolin/100 μM IBMX (F/I). B, amiloride-sensitive Iₑ shown as percentage of control (100%) before and after treatment with 10 μM forskolin/100 μM IBMX (F/I) and after a 30-min pre-treatment in culture with either 1 μM KT5720 or 100 μM Genistein, or both, prior to treatment with 10 μM forskolin/100 μM IBMX (F/I). *, significantly different from control, p < 0.05; **, significantly different from control, p < 0.01; ***, significantly different from forskolin/IBMX, p < 0.05. Results are shown as mean ± S.E.
Statistical analysis was carried out using Student’s unpaired or paired t-tests where p values of < 0.05 were considered significant. Results are presented as mean ± S.E.

RESULTS

Effect of Forskolin on Ion Transport Processes across H441 Cell Monolayers—Application of forskolin/IBMX to H441 cell monolayers was carried out before and after application of amiloride (Fig. 1A, i and ii). Forskolin induced a significant rise in $I_{\text{sc}}$ from $10.9 \pm 1.6 \mu A \text{ cm}^{-2}$ to $14.1 \pm 2.0 \mu A \text{ cm}^{-2}$, p < 0.001, n = 10, that was inhibited by amiloride
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FIGURE 3. Representative immunoblot (n = 3) and Western blot (n = 10) of EGFP proteins in H441 cell clones. A, anti-EGFP was used to immuno-precipitate EGFP proteins from total protein lysates (-0.5 mg) of control H441, GFP7, and both EGFP-hαENaC clones (αC3-3 and αC3-5). Immuno-precipitates were fractionated on denaturing gels and Western blotted with anti-αENaC. Immuno-stained proteins representative of full-length (clone αC3-3) and truncated (clone αC3-5) EGFP-hαENaC (~121 kDa and ~48 kDa, respectively), are indicated by the arrows to the left. No specific products were detected in either cells expressing EGFP alone (GFP7) or un-transfected H441 cells. The additional arrow indicates a nonspecific band representative of IgG from the antiserum used in the immunoprecipitation. This blot was repeated three times with similar results. B, total protein lysates (30 μg) from control H441 and GFP7 were subject to Western blot analysis and immuno-stained with anti-EGFP. A protein band of the appropriate size (~27 kDa) expressed in GFP7 clone is indicated on the left. C, light microscopy image of confluent αC3-3 cells grown on plastic culture dishes. Arrows indicate areas of uplifted cells (domes).

to 2.8 ± 0.4 μA cm⁻², p < 0.001, n = 10. Pre-treatment with amiloride (control Iₐₗ, 9.7 ± 1.9 μA cm⁻²; amiloride, 2.6 ± 1.1 μA cm⁻², p = 0.01, n = 5) prevented the forskolin-induced rise in Iₐₗ (1.5 ± 0.3 μA cm⁻², p = 0.01, n = 3). The amiloride-sensitive component of Iₐₗ (Iₐₗ₉) was significantly higher after forskolin treatment (p < 0.05, n = 10). In all experiments, there was no further visible blockade after basolateral application of ouabain (data not shown).

Role of PKA and PTK in Regulating Ion Transport Processes across H441 Cell Monolayers—Forskolin induced a 34 ± 7% rise in amiloride-sensitive Iₐₗ (Iₐₗ₉) from 9.7 ± 1.3 μA cm⁻² to 13.2 ± 1.8 μA cm⁻² (p < 0.05, n = 8) in H441 cell monolayers. This forskolin-stimulated rise in Iₐₗ₉ was significantly blocked by Genistein to 71 ± 8% of control currents (p < 0.01, n = 5) (Fig. 1B). KT5720 exhibited a more profound inhibition of Iₐₗ₉ to 58 ± 5% of control currents (p < 0.01, n = 4). Application of Genistein and KT5720 together also inhibited the forskolin-stimulated rise in Iₐₗ₉ to 80 ± 22% (p < 0.05, n = 3) (Fig. 1B). Together these results indicate that both PKA- and PTK-dependent pathways are involved in mediating the forskolin-stimulated increase in Iₐₗ₉ in H441 cells, but their effect was not additive. Interestingly, KT5720 also significantly inhibited basal Iₐₗ in H441 cells (control: 9.7 ± 1.3 μA cm⁻²; KT5720: 4.6 ± 0.9 μA cm⁻², p < 0.05, n = 4), whereas Genistein was without significant effect (n = 4).

Stable Expression of EGFP-hαENaC in H441 Cells and RT-PCR—H441 cells were transfected with plasmids encoding EGFP-hαENaC (pC3) or EGFP alone (pEGFP-C3). Geneticin-resistant clones were expanded and screened for EGFP fluorescence. Three clones (αC3-3, αC3-5, and GFP7) were chosen based on their homogeneity in terms of EGFP-associated fluorescence. RT-PCR amplification of the structural protein β-actin was carried out on all clones to control for RNA quality and abundance. A 450-bp β-actin product was amplified from αC3-3, αC3-5, and GFP7 at similar yield. RT-PCR analysis using primers to amplify sequences from EGFP (β₂-sense) to the C-terminal of the hαENaC sequence (HCR), from β₂-sense to an internal hαENaC sequence (ASHR) (reverse), and from this site (ASH) (forward) to HCR was used to confirm expression of full-length EGFP-hαENaC mRNA in the clones (Fig. 2, A and C). Products of the correct predicted size were amplified from the plasmid construct pC3 and the αC3-3 clone but not from αC3-5, GFP7 (Fig. 2A), or untransfected cells (data not shown). Amplification of the full-length EGFP-hαENaC product from β₂-sense-HCR resulted in a lower product yield from clone αC3-3 compared with that of the two shorter products (β₂-sense-ASHR and ASH-HCR) that encompass either half of the construct. The yield of this product was also reduced when amplified from the control plasmid pC3. Therefore, this finding most likely reflects a reduced efficiency of amplification rather than a reduced abundance of full-length mRNA product in αC3-3. RT-PCR using primers to amplify EGFP-coding region sequences showed that EGFP was present in GFP7 and αC3-3 cell clones (Fig. 2B). No transcripts for EGFP were detected in untransfected control cells. Taken together these data show that full-length EGFP-hαENaC mRNA was expressed in the αC3-3 clone but a genetically truncated product containing at least, EGFP, was present in the αC3-5 clone (Fig. 2C). GFP7, as expected expressed mRNA for EGFP alone.

Immunoprecipitation of EGFP-hαENaC Proteins from H441 Cell Clones—To confirm that EGFP-hαENaC proteins translated from the RNA transcripts were expressed in the clones, cell lysates (~0.5 mg of total protein) were subjected to immunoprecipitation with anti-EGFP, separated by SDS-PAGE, and Western blotted with anti-αENaC (Fig. 3A). The anti-αENaC epitope is located at the N terminus of the hαENaC protein (Fig. 2C). Blots (n = 3) revealed a distinct band of ~121 kDa in the lane representing the αC3-3 clone containing full-length EGFP-hαENaC, whereas a smaller band of ~48 kDa was evident in the αC3-5 clone representing truncated EGFP-hαENaCt. Together with RT-PCR data we have deduced that this clone contains ~157 amino
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FIGURE 4. Expression of full-length EGFP-haENaC in H441 cells alters ion transport processes across cell monolayers. A, spontaneous transepithelial short circuit current (Isc) (shaded bars) and amiloride-sensitive Isc (I\textsubscript{amiloride}) (open bars) across control H441, GFP7, αC3-3, and αC3-5 monolayers. B, forskolin-induced amiloride-sensitive Isc (ΔI\textsubscript{amiloride}) across H441, GFP7, αC3-3, and αC3-5 monolayers, measured by apical application of 10 \( \mu \)M amiloride before or after treatment with 10 \( \mu \)M forskolin/100 \( \mu \)M IBMX. Results are presented as forskolin-stimulated ΔI\textsubscript{amiloride} minus basal I\textsubscript{amiloride}, * significantly different from control H441, p < 0.05; #, significantly different from GFP7, p < 0.02. Results are shown as mean ± S.E.

layers than in untransfected cells, but we could not attribute significance to these findings (p = 0.1, n = 5). It is possible that transfection procedures and/or the very high level of EGFP protein present in these cells could impede translocation of endogenous ENaC subunits to the membrane.

**Forskolin Stimulation Increased the Density of haENaC at the Apical Membrane of H441 Cell Monolayers**—To explore the basis of the ~50% increase in I\textsubscript{amiloride} seen in αC3-3 cells in response to forskolin treatment, apical biotinylation was performed on polarized cell monolayers. Increased abundance of endogenous haENaC (~93 kDa) accessible to biotin labeling at the apical surface was observed in both αC3-3 and GFP7 clones following a 40-min treatment with forskolin (n = 2) (Fig. 5A). Full-length EGFP-haENaC subunits (~121 kDa) also increased in abundance at the apical membrane of αC3-3 cells. The increase in mean apical abundance, measured by densitometry scanning of the immunostained proteins, is shown in Fig. 5B (n = 2). Although both proteins were also detected in non-biotinylated protein samples, no changes in abundance were observed after forskolin treatment. Thus, the forskolin-induced increase in I\textsubscript{amiloride} in untransfected H441 and GFP7 monolayers could be attributed to the translocation of endogenous haENaC to the membrane (Figs. 4A, 5A, and 5B).

Our data indicate that the additional forskolin-induced I\textsubscript{amiloride} in αC3-3 cell monolayers is due, in part, to the combined insertion of both endogenous and EGFP-haENaC channel subunits in the apical membrane. Blots were re-probed with anti-β-actin, and no proteins of the expected size were detected in the biotinylated protein samples. β-Actin was detected in the non-biotinylated intracellular fraction, which was run in parallel and in total protein extracted from H441 cells.

**Real-time Trafficking of Full-length EGFP-haENaC in H441 Cells Monolayers**—Real-time confocal microscopy showed that EGFP-haENaC subunits were distributed throughout the cytoplasm in polarized, living, clone cell monolayers. Green fluorescence was not detected in control H441 cells, and expression of EGFP alone appeared “ghost-like” throughout the cell (data not shown).

We used rhodamine-WGA, which binds to glycosylated proteins, as a marker for plasma membranes on fixed H441 cells to further explore the distribution of EGFP-haENaC before and after stimulation with forskolin/IBMX. We found that WGA binding was heterogeneous, and many of the cells in the transfected clones did not efficiently bind WGA (Fig. 6A, i). However, some co-localization of EGFP fluorescence with WGA was detected. The distribution of EGFP-haENaC in αC3-3 was similar to that of endogenous ENaC immunostained with FITC-anti-aENaC (Fig. 6A, i). Translocation of EGFP-haENaC toward the apical membrane after stimulation with forskolin/IBMX was seen in fixed αC3-3 cells. This was similar to the translocation of immunostained haENaC in untransfected control cells (Fig. 6A, i and ii). Unfortunately, we were unable to obtain WGA staining of sufficient intensity to provide good quality pictures for live cell imaging. However, the uppermost z-plane of monolayers in which WGA and EGFP-haENaC resided could be identified (see “Experimental Procedures” and Fig. 6A). Representative live cell images of cells expressing both the full-length (αC3-3) and truncated (αC3-5) EGFP-haENaC can be seen in Fig. 6B. In both the αC3-3 and αC3-5 clones, 100% of cells exhibited EGFP-associated fluorescence when compared with non-transfected control H441 cells. Fluorescence intensity varied between individual cells, but no obvious differences in the distribution of the EGFP-haENaC proteins were observed between the two clones. Only those cells expressing the full-length product (αC3-3) responded to forskolin treatment and translocated EGFP-haENaC toward the apical z plane (Fig. 6, A–C). In...
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**FIGURE 5.** Representative Western blot of hαENaC proteins expressed at the apical surface of cell monolayers. A, biotinylated proteins and non-biotinylated proteins (50 μg) from αC3-3 and GFP7 monolayers, untreated (−FI) or treated with 10 μM forskolin/100 μM IBMX for 40 min (+FI) and total cell homogenate from untransfected H441 cells (50 μg) subject to Western blotting with anti-αENaC (αENaC). Protein bands representative of EGFP-hαENaC (~121 kDa) and endogenous hαENaC (~93 kDa) are indicated by the arrows to the left. Blots were also immunostained with anti-β-actin (β-actin) as a negative control for surface biotinylation (~48 kDa). B, bar chart showing mean density of immunostained biotinylated EGFP-hαENaC and hαENaC in untreated (open bars) or forskolin/IBMX-treated (filled bars) αC3-3 and GFP7 cells (n = 2).

a single field of view, cells exhibiting both high and low levels of EGFP-associated fluorescence were seen to translocate. The mean fluorescence intensity of the apical plane for 11 individual cells in the αC3-3 clone monolayer increased by 108% (p < 0.001, n = 11) after stimulation with IBMX/forskolin (Fig. 7A). In contrast, there was no significant difference in apical fluorescence of αC3-5 (Fig. 7A) or GFP7 cells (data not shown) after forskolin stimulation. Overall, there was a decrease in fluorescence of ~2% in αC3-5 cells over time that could reflect photobleaching or channel recycling (Fig. 7A). The increased movement of EGFP-hαENaC to the apical membrane in αC3-3 monolayers was consistent with the increased density of biotinylated EGFP-hαENaC at the apical membrane after treatment with forskolin/IBMX and was maximal after 30 min.

**Forskolin-stimulated Movement of EGFP-hαENaC Is Blocked by Brefeldin A**—Pre-treatment of αC3-3 for 30 min with Brefeldin A, a vesicular trafficking inhibitor, significantly inhibited the movement of EGFP-hαENaC to the apical membrane in response to forskolin treatment (p < 0.05, n = 3) (Fig. 7, B and C). The EGFP fluorescence intensity after forskolin treatment only increased by 2.5% compared with 108% seen in control αC3-3 monolayers (Fig. 7C). These data confirm that the increase in EGFP-hαENaC at the apical membrane in response to forskolin was via a process that involves vesicle trafficking to the apical membrane.

**Forskolin-stimulated Movement of EGFP-hαENaC Is through a PTK-dependent Pathway**—The PKA-inhibitor KT5720 partially blocked forskolin-stimulated trafficking of EGFP-hαENaC subunits to the apical membrane (p < 0.01, n = 15) but there was still a 38% increase in fluorescence after stimulation with forskolin (Fig. 7, B and C). In contrast, translocation of EGFP-hαENaC to the apical surface was practically abolished in the presence of the PTK-inhibitor Genistein (p < 0.001, n = 15), and the fluorescence only increased by 3.6% after forskolin-stimulation (Fig. 7, B and C).

Forskolin-stimulated Trafficking of EGFP-hαENaC Is Associated with a Decrease in Cell Height—As we could clearly demonstrate trafficking of EGFP-hαENaC (αC3-3) subunits in response to forskolin stimulation, we investigated potential mechanisms underlying this effect. We found that forskolin stimulation was associated with a decrease in cell height of 7.1 ± 0.4 μm (p < 0.001, n = 15). The average cell height within the monolayer was 29 ± 2 μm. This effect was most likely related to cell volume changes and was indicative of shrinkage of these cells (Fig. 8A). We observed the changes in cell height to be gradual (1–2 μm per 5 min) over the time course of the experiment, reaching a maximum at 30 min. This correlated with the time-dependent rise in fluorescence appearing at the apical membrane of these cells (R² = 0.97). The forskolin-induced reduction in cell height was not significantly affected by pre-treatment with Brefeldin A (n = 3). Genistein also did not significantly block the forskolin-induced decrease in cell height (n = 12), although in these experiments the change was smaller than that of forskolin only treated cells (4 ± 0.3 μm). There was no significant change in cell height after preincubation with KT5720 followed by forskolin stimulation (p < 0.001, n = 15) indicating that PKA mediated this effect (Fig. 8C). A decrease in cell height was also observed in αC3-5 and GFP7 cells (p < 0.001, n = 12 and p < 0.05, n = 5), respectively, but these were much smaller than those in αC3-3 cells (Fig. 8B). No effects on cell
height were observed after preincubation with Genistein, KT5720, or Brefeldin A (n = 3) in the absence of forskolin.

DISCUSSION

H441 cells are derived from adult bronchiolar epithelium and are a predominantly Na\(^{+}\) absorptive epithelial cell line, which expresses mRNA and protein for the three subunits of ENaC (15, 25). Our data confirm that the amiloride-sensitive component of short circuit current (I\(_{\text{amiloride}}\)) in these cells comprises at least 80%. In accordance with other studies, we have shown that elevation of cAMP with forskolin, together with IBMX, a cAMP-dependent phosphodiesterase inhibitor (22), functionally increases amiloride-sensitive I\(_{\text{amiloride}}\) in H441 human adult lung epithelial cells (14, 16, 25) within 40 min. As this process is blocked by Brefeldin A, a vesicle-trafficking inhibitor, the route of action of cAMP has been postulated to involve trafficking of endogenous ENaC channels to the apical membrane (14, 16, 25). Thomas and colleagues showed that this signaling event was via a PKA-dependent pathway (14, 16, 25). However, in rat FDLE cells, forskolin stimulated I\(_{\text{amiloride}}\), through a PTK-dependent pathway (17, 18, 26). Our results show that both PKA and PTK pathways were involved in mediating the effects of forskolin on I\(_{\text{amiloride}}\) in H441 cell monolayers. We also found that PKA was involved in regulating basal levels of I\(_{\text{amiloride}}\). This effect of PKA and lack of involvement of PTK on basal I\(_{\text{amiloride}}\) has been described in rat FDLE cells, although Thomas et al. (17) did not report it in H441 cells.
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To investigate the dynamic changes in human αENaC subunit distribution in H441 cell monolayers under these functional responses to forskolin, we generated EGFP-hαENaC fusion proteins with EGFP expressed at either the C or N termini of hαENaC. A similar approach was used to study the expression of Xenopus αENaCs expressed in kidney A6 cells (19), but this is the first report to show expression of EGFP-labeled human αENaC in lung cells. We generated three H441 cell clones, one expressing EGFP alone (GFP7) and two others expressing EGFP-hαENaC. One of these clones (αC3-3), expressed a full-length mRNA and chimeric protein, but the other (αC3-5) was genetically truncated, containing only EGFP and the first ~157 amino acids of hαENaC (EGFP-hαENaCt). Both EGFP-hαENaC proteins were expressed at low levels in the cell compared with EGFP alone (as indicated by fluorescence and the amount of protein required to obtain a signal from immunoblots; 0.5 and 30 mg, respectively). Similar to the findings of Blazer-Yost et al. (19), we have shown that a full-length chimeric protein produced functional channels in H441 cells as it augmented amiloride-sensitive I_{amiloride} to double that of control cells. In addition, we also observed the formation of domes when αC3-3 cells were cultured on plastic. This is indicative of Na⁺ transport coupled with osmotic water movement from the apical to the basolateral side of the epithelial cell monolayer. Dome formation in lung and H441 cell monolayers has been reported to be associated with increased I_{amiloride} (27). Thus, the elevated amiloride-sensitive Na⁺ transport present in the αC3-3 clone is a likely explanation for this phenomenon.

We have also shown that full-length EGFP-hαENaC protein and endogenous hαENaC are expressed predominantly in the cytoplasm of the αC3-3 clone. Confocal microscopy and surface biotinylation revealed that both EGFP-hαENaC and endogenous αENaC proteins were present in the apical membrane and both increased in abundance at the cell surface 40 min after forskolin stimulation. As forskolin-stimulated I_{amiloride} was greater than that of GFP7 and untransfected cells, we were confident that the fusion protein was contributing to the formation of functional channels responsive to forskolin stimulation. Interestingly, Blazer-Yost and colleagues (19) refrained from tagging the N terminus of Xenopus αENaC, because this region was thought to be important for targeting the protein to the apical membrane (28). Deletion of amino acids in the N terminus of mouse αENaC has been shown to reduce channel activity and mildly decrease channel protein at the membrane when co-expressed with β- and γENaC in Xenopus oocytes (29). It has also been proposed that trafficking signals are contained both before and after the first 535 amino acids of rat αENaC (28), and proline-rich sequences in the C-terminal of rat αENaC have been shown to be important for localizing ENaC to the apical membrane (31). Consequently, both N- and C-terminal regions of αENaC may be important for trafficking processes. Linking EGFP to either terminal could potentially prevent the access or binding of regulatory proteins to these regions and modify membrane targeting. To our knowledge there are no reports that indicate that EGFP has adverse effects when placed on the C or N termini of αENaC. In support of this, our data show that tagging the N terminus of αENaC did not inhibit function, membrane localization, or forskolin-induced trafficking of the subunit.

Surprisingly, I_{amiloride} and I_{amiloride} in the αC3-5 clone were significantly lower than that of control cells, inferring that the truncated EGFP-hαENaC had detrimental effects on ion transport processes in H441 cells. Interestingly, Bonny et al. (32) reported that a similar hαENaC mutant bearing 143 amino acids of the N terminus, in association with hβγENaC, gave amiloride-sensitive currents that were only 0.1% of wild-type αβγENaC when expressed in Xenopus oocytes. Thus, expression of EGFP-hαENaCt in H441 cells could result in the competitive formation of non-functional ENaC channels in this clone.

It has been reported that the stimulatory action of elevated cAMP on lung epithelial amiloride-sensitive Na⁺ transport is mediated through three independent mechanisms; increased trafficking and insertion of ENaC proteins in the membrane, decreased retrieval of proteins, and increased channel activity (14, 16, 25, 33, 34). Using the technique of real-time confocal microscopy on live, polarized, intact H441 cell monolayers we observed that forskolin increased trafficking of full-length EGFP-hαENaC (αC3-3) from the cytoplasm to the apical membrane in a Brefeldin A–sensitive manner. In light of the fact that αENaC subunits are essential for channel pore formation and are capable of forming channels independently of β and γ (2), the recruitment of additional αENaC proteins to the apical membrane would result in an increase in the number of functional channels and a concomitant rise in I_{amiloride}, as we observed in these cells. The time course of insertion was maximal after 30 min, which was longer than that observed for maximal function (20–30 min). However, the confocal studies were carried out at room temperature as opposed to 37 °C in the Ussing chamber experi-
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ments. While it is likely that EGFP-hαENaC is associated with β- and γ-subunits in the apical membrane (because they are endogenously expressed in H441 cells), ENaC subunit proteins could be independently trafficked to the apical membrane (21). Further work will be required to investigate these possibilities. Because we did not observe trafficking of EGFP-hαENaC in αC3-5 monolayers, it seems likely that the truncated channel protein is not effectively translocated to the membrane, and this could underlie the loss of function we and others have described (32).

Genistein completely abolished trafficking of EGFP-hαENaC to the apical surface. Genistein is classically used as a broad spectrum PTK inhibitor, and it has little effect on PKA or protein kinase C especially when used at 100 μM as in our study. Both Genistein and another PTK inhibitor, tyrophostin A23, have been shown to have similar effects on PTK-stimulated Na+ transport in rat distal lung cell monolayers (35). Thus, we conclude that the recruitment of EGFP-hαENaC to the membrane was predominantly via a PTK-dependent pathway, because KT5720 only blocked trafficking by 60%. When we compared these results to the effects of the same inhibitors on Iamiloride in control H441 cells, both KT5720 and Genistein inhibited forskolin-stimulated Iamiloride and KT5720 significantly blocked basal Iamiloride. Together, these results indicate that PKA regulates amiloride-sensitive Itr, through additional mechanisms to that of trafficking of EGFP-hαENaC. This could include activation of channels already in the membrane of H441 cells (16) and down-regulating retrieval of ENaC from the apical membrane (34). We have shown that the forskolin-stimulated trafficking of EGFP-hαENaC (αC3-3) across cell monolayers was accompanied by a significant decrease in cell height within the monolayer. We also determined smaller changes in cell height in αC3-5 and GFP7 cells inferring that the effect of forskolin on cell volume was also present in cells that expressed and translocated endogenous hαENaC. It also appeared that the degree of cell shrinkage was related to the activity of ENaC in the membrane as αC3-3 monolayers exhibited higher basal amiloride-sensitive Itr, compared with αC3-5 and GFP7. Forskolin has been reported to cause a rapid apical membrane depolarization of H441 monolayers via activation of ENaC channels already in the membrane (36). If membrane depolarization via ENaC initiates mechanisms that lead to cell shrinkage and translocation of new channels to the membrane, this could potentially provide a link between the number of channels in the membrane and the degree of shrinkage we see in our cells. This hypothesis however, remains to be tested.

The change in cell height was maximal at 40 min, similar to the time point where a maximal change in Itr and translocation of EGFP-hαENaC were observed. However, although Brefeldin A blocked the forskolin-induced rise in amiloride-sensitive Itr in H441 cells (25) and trafficking of EGFP-hαENaC to the apical membrane in our study, this inhibitor was without effect on cell shrinkage. This observation indicates that cell shrinkage is not a consequence of trafficking of hαENaC to the membrane, but rather, cell shrinkage stimulated incorporation of EGFP-hαENaC into the apical membrane. The observed reduction in cell height in αC3-3 monolayers was inhibited by KT5720 but not Genistein indicating that PKA mediated this effect. These findings are supported by Hosoi and co-workers (26) who found that terbutaline (β2-agonist) induced cAMP-accumulation and a PKA-dependent, amiloride-independent cell shrinkage in adult alveolar Type-II cells. Furthermore, Niisato et al. (17) hypothesized that forskolin induced cell shrinkage in FDLE cells and activated PTK, which consequently induced recruitment of amiloride-sensitive channels to the membrane (17). As the inhibition of PTK had no effect on cell shrinkage but inhibited trafficking of EGFP-hαENaC to the membrane, and because inhibition of PKA affected both pathways, our data indicate that these kinases act in sequence. This is further supported by our finding that KT5720 and Genistein inhibited the forskolin-induced rise in Iamiloride, but their effect was not additive. We speculate that forskolin induces a PKA-dependent reduction in cell volume, which activates PTK and stimulates translocation of EGFP-hαENaC to the H441 cell apical membrane. Both Hosoi et al. (17) and Niisato et al. (26) have implicated Cl−secretion, because a mechanism mediating forskolin induced cell shrinkage. A forskolin-induced membrane depolarization attributable to Cl−secretion and the presence of cystic fibrosis transmembrane regulator (CFTR) have been described in H441 cells (16). Genistein has been reported to potentiate Cl−secretion through CFTR (30). However, any augmentation on PTK activity and subsequent trafficking would be simultaneously inhibited by this agent. Thus, the role of Cl−secretion in this response remains to be tested.

In summary, we have generated stable human H441 cell clones, one of which expresses a full-length functional EGFP-hαENaC. Using a combination of functional and biochemical approaches we show for the first time that forskolin treatment of H441 cells induces activation of PKA to provoke dynamic changes in cell height within a transporting monolayer, which are indicative of cell shrinkage/volume changes. We propose that this process evokes activation of PTK and stimulates translocation of αENaC subunits to the apical membrane. Our demonstration that cell shrinkage induces translocation of the pore forming α-subunit of ENaC to the apical membrane of epithelial monolayers provides an underlying mechanism for the forskolin-induced increase in Na+ transport we and others observe. Furthermore, the clones we have generated will provide useful tools to further study the role of adrenaline in regulating the translocation and activation of ENaC subunits in the lung epithelial cell membrane and the mechanisms that underlie the regulation of lung fluid absorption and homeostasis in the newborn and adult lung.

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