Insulin-stimulated trafficking of ENaC in renal cells requires PI 3-kinase activity

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The A6 model renal epithelial cell line has been used to study the mechanisms by which insulin stimulates Na⁺ transport. At the level of the apical membrane, blocker-induced noise analysis has indicated that the increased transport is due to an increase in the number of active channels in the membrane, which could be due to either an activation of quiescent channels or an insertion of channels from an intracellular pool (3).

Intracellularly, the pathway linking the insulin receptor on the basolateral membrane to increased channel number on the apical membrane is dependent on the activity of phosphatidylinositol 3-kinase (PI 3-kinase). The product of this enzymatic reaction, phosphatidylinositol 3,4,5-trisphosphate (PIP₃), is formed within 1 min of insulin stimulation (14). An inhibitor of the enzyme, LY-294002, blocks both basal and insulin-stimulated Na⁺ transport by preventing the insulin-stimulated increase in active channel number in the apical membrane (3, 13).

Formation of PIP₃ initiates a signal transduction cascade involving lipid and protein kinases and phosphatases, and one of these, SGK (serum, glucocorticoid-induced kinase) has been implicated in the regulation of ENaC. SGK is an aldosterone-induced protein in cultured renal cells (12) and is required for basal as well as steroid and peptide hormone-mediated ENaC activity in A6 cells (6). A6 cells stably transfected with wild-type SGK to supplement the endogenous levels of active channels in the membrane, which could be due to either an activation of quiescent channels or an insertion of channels from an intracellular pool (3).

Alternatively, insulin-mediated stimulation of PI 3-kinase and the resulting formation of PIP₃ may have more direct actions on ENaC. Patch-clamp techniques have been used to excise portions of the A6 cell apical membrane containing ENaC to directly assess the role of the phosphoinositides on channel kinetics. With the use of similar experimental design, two recent reports reached somewhat different conclusions (9, 19). Yue et al. (19) demonstrated that the ENaC in the isolated
membrane patches showed an increase in $N P_o$ ($N$ is channel number, $P_o$ is open probability) in response to phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and GTP (or G$_{o,i,3}$). The channels in the patches were not altered by the addition of PIP$_3$. Conversely, Ma et al. (9) found that addition of PIP2, PIP$_3$, or phosphatidylserine (PS) prevented channel rundown in the excised membrane patches.

It is important to note that in both studies the cells were pretreated with a high concentration of aldosterone for prolonged periods. We have previously shown that aldosterone treatment for as little as 20 min increases endogenous PIP$_3$; therefore, the cells used in the patch-clamp experiments may already have maximally effective amounts of PIP$_3$. It is also unclear how the formation of a gigahm seal during patch formation may alter dynamic lipid interactions within the plasma membrane or the trafficking of proteins such as ENaC to the cell surface in response to hormonal stimuli.

It is possible that multiple components of the phosphoinositide pathway are involved in activation as well as “deactivation” of ENaC. These processes may involve changes in channel number on the plasma membrane, changes in channel kinetics, and/or changes in phosphorylation of intermediates such as NEDD4 (neuronal precursor cell developmentally downregulated protein 4), which will secondarily regulate channel turnover.

To fully understand the pathways involved in insulin-stimulated Na$^+$ transport, several important issues need to be resolved. Two different electrophysiological techniques, patch clamp and blocker-induced noise analysis, have been used to study single-channel kinetics. However, even in the same model cell line there is some controversy as to whether insulin causes an increase in active channel number or an increase in $P_o$(3, 11). In the current studies, we have taken an independent approach and used enhanced green fluorescent protein (EGFP)-labeled ENaC subunits to determine whether the channel location varies in response to insulin stimulation.

We have also sought to determine the localization of PI 3-kinase in both stimulated and nonstimulated A6 cells. To predict function, it is important to establish whether the major localization of PI 3-kinase is near the basolateral surface, where the enzyme links the activation of the insulin receptor and insulin receptor substrate (IRS) to a downstream signaling complex, and/or whether the PI 3-kinase is more immediately and directly involved in active ENaC expression and kinetics at the apical membrane. Furthermore, it would be interesting to know whether PI 3-kinase and ENaC colocalize in either stimulated or nonstimulated A6 cells and, if so, to establish whether this interaction is blocked by treatment with LY-294002, an inhibitor of PI 3-kinase activity.

Studies presented here show that insulin stimulation results in a translocation of ENaC to the lateral and apical membrane. In addition, insulin stimulation results in colocalization of the channel and PI 3-kinase. Although colocalization of PI 3-kinase and ENaC is not blocked by treatment with the PI 3-kinase inhibitor LY-294002, the subcellular translocation of the ENaC-PI 3-kinase complex to the lateral plasma membrane is inhibited and the subsequent translocation of ENaC to the apical membrane is blocked.

**MATERIALS AND METHODS**

**Cell culture.** The A6 cell line, originally derived from the kidney of *Xenopus laevis*, was obtained from American Type Culture Collection (Rockville, MD). The parental A6 cell line was grown at 27°C in a modified DMEM (no. 91-5055EC; Life Technologies, Gaithersburg, MD) supplemented with 25 U/ml penicillin, 25 μg/ml streptomycin, and 10% newborn calf serum in a humidified incubator gassed with 5% CO$_2$. The αENaC-EGFP A6 cell clones (1) were grown in the same medium containing 1 mg/ml Geneticin (Life Technologies). The cells were subcultured onto 24-mm Transwell inserts (Costar, Cambridge, MA) as previously reported (2) for at least 14 days to achieve confluence. Cells grown on Transwell supports were used in all of the reported studies. At the time of seeding onto the Transwell filters, Geneticin was removed from the tissue culture media of the αENaC-EGFP clones.

**Electrophysiology.** Short-circuit current (SCC) techniques were used to determine net ion flux across high-resistance, confluent epithelia as reported in previous studies (2, 6, 14). Insulin (100 nM; Lilly Research Laboratories) was added to...

![Fig. 1. Confocal images of control (non-insulin treated) A6 cells stably expressing enhanced green fluorescent protein (EGFP)-epithelial Na$^+$ channel (ENaC) (green) and counterstained with anti-phosphatidylinositol 3-kinase (PI 3-kinase) p85 antibody (red) and nuclear 4,6-diamidino-2-phenylindole (DAPI) stain (blue). The image in A is within the first micrometer of the top (apical) portion of the cells; the image in B is an optical slice through the approximate middle of the cells.](http://ajpcell.physiology.org/content/284/6/A1646/F1.large.jpg)
the serosal bathing media, and amiloride (10⁻⁵ M) and LY-294002 (50 μM; Biomol Research Laboratories, Plymouth Meeting, PA) were added to the apical media. Amiloride was added at the terminal point of each experiment to indicate the portion of the SCC due to Na⁺ transport via ENaC.

Confocal microscopy and data analysis. αENaC-EGFP A6 cell clones were used for all confocal studies. In the insulin-treated cells, insulin was added to confluent monolayers for 1 min before fixation. The cells were fixed with 3.7% paraformaldehyde in PBS for 5 min, washed, and permeabilized with 0.25% NP-40 for 15 min. To visualize PI 3-kinase, we incubated the permeabilized monolayers overnight in a 1:100 dilution of an anti-PI 3-kinase p85 subunit antibody (Upstate Biotechnology, Lake Placid, NY) and subsequently with a 1:200 dilution of an anti-PI 3-kinase p85 subunit antibody (Upstate Biotechnology, Lake Placid, NY) and subsequently with a 1:100 dilution of a 1:200 dilution of a fluorescent goat anti-rabbit IgG secondary antibody (Alexa 568; Molecular Probes, Eugene, OR) for 30 min in a humidified chamber. If either the primary or secondary antibodies were omitted during the procedure, no fluorescence corresponding to the PI 3-kinase was detected.

Images were collected with a Bio-Rad MRC1024-UV confocal system (Bio-Rad, Hemel Hempstead, UK) using a ×40 Plan Apo, 1.3 NA oil-immersion objective on a Nikon Diaphot 200 inverted microscope. The green and red fluorescent images were collected sequentially with LaserSharp 3.1 acquisition software (Bio-Rad). Images were collected as a stack in the PIC format.

Green fluorescent molecules were excited with a krypton-argon laser at 488 ± 10 nm, and emission at 522 ± 17 nm was detected with a photomultiplier tube (PMT). Red fluorescent molecules were excited with a 568-nm krypton-argon line, and emission >585 nm was detected with a PMT.

Visualization and reconstruction of the image stacks were done with Imaris software (version 3.1.1; Bitplane, Zurich, Switzerland). Colocalization analysis was performed with LaserPix (Bio-Rad), a program developed from algorithms published by Manders et al. (10). In this type of colocalization calculations, the coefficients are independent of the relative intensities of the green and red channels.

RESULTS

Insulin stimulates ENaC trafficking to the lateral and apical plasma membranes. We have previously prepared ENaC subunit chimeras containing EGFP. The EGFP was added to the carboxyl terminus of the α-subunit and to the amino terminus of the β-subunit. Both α- and β-ENaC-transfected cell lines exhibit normal Na⁺ transport responses to insulin (1). An αENaC-EGFP was used in the current studies.

It is important to note that in all clones the EGFP subunit expression shows cell-to-cell variability (Fig. 1). In a confluent culture, some cells will express higher levels of the fluorescently labeled subunit than neighboring cells. Every attempt was made to establish these as single-cell clonal lines, and multiple clones have been examined with consistent results. Therefore, we feel that this nonuniform expression is a normal, physiologically relevant finding. For this reason, the microscopy and colocalization were performed on single cells within confluent monolayers.

In previous studies using the stably transfected cell lines expressing EGFP-labeled subunits, we were unable to detect ENaC labeling within the nuclear space (1). In the current studies, we examined thin optical sections of both control and insulin-stimulated cells to determine whether PI 3-kinase and the nuclear stain showed colocalization (data not shown). No significant amount of PI 3-kinase was found in the nucleus.

To determine whether insulin stimulation caused a translocation of ENaC to the cell membrane, we treated cells with 100 nM insulin for 1 min before paraformaldehyde fixation. Matched control cells were grown under identical conditions in the same six-well Transwell culture plate. Figure 2 depicts the localiza-

![Fig. 2. Confocal imaging of EGFP-ENaC in control (A) and insulin-stimulated (B) A6 cells. The imaging was performed on 1–2 cells within a confluent monolayer grown on permeable supports. The top left panel of each triad represents an xy optical slice taken at approximately the midpoint of the cell. To the right of the xy image is the yz cross section of the entire cell with the apical cell membrane toward the right. Below the xy image is the xz cross section with the apical membrane at the bottom-most portion of the image.](http://ajpcell.physiology.org/DownloadedFrom/10.1152/ajpcell.00119.2002)
tion of ENaC in representative control and insulin-treated cells. Figure 2 shows three different perspectives (xy, xz, and yz planes) of the same image. Serial confocal sections containing these cells are shown in Fig. 3 (control) and Fig. 4 (insulin treated). In the control cells, the ENaC is distributed throughout the cell. Conversely, after insulin stimulation, ENaC is concentrated at the lateral and apical surfaces.

**Colocalization of ENaC and PI 3-kinase before and after insulin stimulation.** The montages in Figs. 3 and 4 show optical slices of control and insulin-treated cells taken in 1-µm steps starting at the apical membrane. The top panels show the localization of the PI 3-kinase (red), the middle panels show the localization of EGFP-ENaC (green), and the bottom panels show the two fluorophores imaged together. The cells shown in Figs. 3 and 4 are representative of multiple cells that were imaged in this manner. After insulin stimulation, there appears to be more EGFP-ENaC in the apical and lateral portions of the cells. In addition, there is an enhanced colocalization of the PI 3-kinase and ENaC (Fig. 4).

To further examine the colocalization occurring after insulin stimulation, and to obtain a relative quantitation, we analyzed individual cells from the areas shown in Figs. 3 and 4 using LaserPix’s colocalization module. The data from each of these analyses are plotted in Fig. 5. The green lines represent the relative amount of ENaC that is colocalized with PI 3-kinase, and the red lines represent the relative amount of PI 3-kinase that is colocalized with ENaC. The PI 3-kinase is much more abundant than the ENaC; therefore, the red and green lines must be interpreted separately. After insulin stimulation, the vast majority of ENaC is associated
with PI 3-kinase. Interestingly, although PI 3-kinase is much more abundant than ENaC, a substantial proportion of the enzyme is colocalized with ENaC. This colocalization does not appear to be at the apical membrane (optical slices 1–3) but, rather, at a site just below the apical membrane. To more carefully visualize site of colocalization, we have provided yz and xz slices in Fig. 6. Figure 6, combined with Figs. 2–5, shows that the major portion of the colocalization happens after insulin stimulation and is found along the lateral membrane.

**Effect of inhibition of PI 3-kinase on insulin-stimulated ENaC trafficking.** LY-294002, a reversible inhibitor of PI 3-kinase, inhibits both basal and insulin-stimulated Na\(^+\) transport in the parental A6 cell line (3, 13, 14). The αENaC-EGFP cell line exhibits a two- to threefold increase in basal ion transport relative to the parental line; therefore, it was important to establish whether this transfected cell line was also sensitive to LY-294002. Electrophysiological studies depicted in Fig. 7 show an enhanced basal transport as previously described (1). This increased basal Na\(^+\) transport was inhibited by 50 μM LY-294002 with a time course similar to that observed in the parental cell line (3, 13). The insulin-stimulated transport was also inhibited by LY-294002, particularly in the first 10 min after insulin stimulation when the untreated cells show the largest increase in transport. This early time
is most applicable because this is the time when we are analyzing the changes in ENaC/PI 3-kinase colocalization.

Inhibition of PI 3-kinase activity does not appear to affect the insulin-stimulated colocalization of PI 3-kinase and ENaC. Figure 8 shows the relative amount of ENaC that is colocalized with PI 3-kinase in 1-μm optical sections starting at the apical membrane. In agreement with the study shown in Fig. 5, there is little colocalization in the untreated (i.e., control) cell. Insulin stimulation for 1 min results in a high degree of colocalization. Pretreatment with LY-294002 did not prevent colocalization of PI 3-kinase and ENaC. Interestingly, LY-294002 alone appeared to stimulate colocalization between the channel and PI 3-kinase; however, the relative magnitude of colocalization occurring in the insulin-stimulated vs. LY-294002-treated cells (in the absence of insulin) suggests that effects of LY-294002 on colocalization are less than the effects of insulin, particularly in the subapical portion of the cell (Fig. 9).

More importantly, LY-294002 inhibits the translocation of the PI 3-kinase-ENaC complex. As indicated in Fig. 9, insulin stimulates an association between ENaC and PI 3-kinase in the lateral membrane, which is in agreement with the data presented in Fig. 6. However, pretreatment with LY-294002 has two striking effects. First, after pretreatment with the inhibitor, the insulin-stimulated colocalization of ENaC and PI 3-kinase is no longer confined to the lateral membrane but appears to be distributed throughout the cell (Fig. 9). Second, in the presence of LY-294002, insulin does not stimulate an insertion of ENaC into the apical plasma membrane (Fig. 9). Although LY-294002 does not inhibit the insulin-stimulated colocalization (Fig. 8), it does inhibit insulin-stimulated Na⁺ transport (Fig. 7), further suggesting that impaired channel function is the result of its inability to translocate to the membrane.

**DISCUSSION**

Using blocker-induced noise analysis, we have previously found that insulin-stimulated Na⁺ transport is associated with an increase in active channel number (3) with no increase in \( P_0 \). These findings have been difficult to reconcile with patch-clamp studies that...
have found an insulin-stimulated increase in $P_{\text{Na}}$ (11). In the current studies, we have used an independent approach to determine the mode of action of insulin on ENaC. EGFP-labeled channels react normally to hormonal stimulation (1), and, as shown in Fig. 2, the channels move to the apical and lateral membrane in response to insulin. Only those channels in the apical membrane will participate in transcellular Na$^+$ transport. The role of the channels that are translocated to the lateral portion of the cell is unknown. One intriguing possibility is that these serve as a reservoir to maintain the enhanced transport in the face of the rapid channel endocytosis that is characteristic of the cell line (16). These findings confirm and extend the previous noise analysis results.

The channel redistribution happens within 1 min of hormonal stimulation, a time frame consistent with the insulin-stimulated increase in PIP$_3$ in A6 cells (14). PIP$_3$ is formed by the action of PI 3-kinase; therefore, we were interested in determining the cellular localization of the enzyme both before and after insulin stimulation. The active enzyme is the dimer of regulatory and catalytic subunits. Regulatory subunit binding stabilizes catalytic subunit activity and is thought to recruit PI 3-kinase to the membrane. An antibody to the regulatory subunit of the enzyme was used to determine cellular localization of PI 3-kinase. By immunofluorescence, PI 3-kinase appears to be uniformly dispersed in the cytoplasm, whereas the nucleus is devoid of the protein. There does not appear to be an enrichment of the enzyme in the vicinity of either the apical or basal plasma membrane. However, the abundance of the PI 3-kinase precludes the detection of insulin-induced trafficking of a small percentage of the total enzyme pool.

To ascertain whether insulin stimulation causes a change in the relative proximity of PI 3-kinase and ENaC, we used immunofluorescence to detect enzyme localization in EGFP-ENaC-labeled cells. The montages in Figs. 3 and 4 are representative of multiple cells that were analyzed by confocal imaging. In all cases it was visually difficult to detect colocalization of ENaC and PI 3-kinase in the control cells, whereas there appears to be intense colocalization at specific areas of the cell after insulin stimulation. From Fig. 4, bottom, it appears that the highest level of colocalization is at the points of cell-to-cell contact. This interpretation is substantiated by examining the $xz$ and $yz$ planes of individual cells (Fig. 6). In the nonstimulated cell, the ENaC and PI 3-kinase do not show substantial colocalization at any point within the cellular cross sections. After insulin stimulation, the ENaC is found on the apical and lateral membranes; however, colocalization of PI 3-kinase with the channel is predominately lateral. A quantitation of the colocalization for each of the 1-$\mu$m optical sections in individual cells substantiates that the major colocalization is not on the apical membrane but, rather, directly below the apical membrane.

Together, these data show that after insulin stimulation, as much as 90% of the ENaC is found in the proximity of PI 3-kinase in some of the optical slices. Interestingly, the highest degree of colocalization is along the lateral membrane, likely at or below the tight junctions.

Our technique for colocalization indicates that the proteins are less than 0.2 $\mu$m apart. At this resolution,
we cannot definitively determine whether the PI 3-kinase and ENaC are in direct contact. However, a physical interaction between the two proteins is possible. ENaC is comprised of three subunits, α, β, and γ, each of which contains putative binding sites for SH3 domains such as that found on the regulatory (p85) subunit of PI 3-kinase. SH3 domains are pseudosymmetric and are thought to bind protein ligands in two opposite orientations (class I, NH₂ to COOH terminus, and class II, COOH to NH₂ terminus) (7, 18). In particular, both the β- and γ-subunits contain the motif COOH-PxPxxP-NH₂, which serves as a class II binding site that can interact with the SH3 domain of the p85 subunit of PI 3-kinase. It is interesting to note that the SH3 binding domain overlaps with the proline-rich PY motifs that are putative binding sites of NEDD4, a negative regulator of ENaC (15). Whether the proteins are physically bound or not, their close proximity after insulin stimulation suggests that the PI 3-kinase-mediated change in lipid composition is important for ENaC trafficking.

We have previously shown that pretreatment with LY-294002 inhibits basal, insulin-stimulated, and aldosterone-stimulated Na⁺ transport (13, 14). Interestingly LY-294002 does not inhibit insulin-stimulated colocalization of ENaC and PI 3-kinase but, rather, changes the intracellular site of the colocalization from the lateral membrane to a diffuse intracellular localization. As a consequence, no ENaC is translocated to the apical membrane.

LY-294002 is a competitive inhibitor of the ATP binding site of PI 3-kinase. Consequently, this inhibitor would not be expected to disrupt a protein-protein interaction between ENaC and PI 3-kinase, although it can prevent translocation of the protein complex. For instance, previous studies have shown that LY-294002 does not affect PI 3-kinase binding to the insulin-receptor substrate 1 protein, although it does prevent translocation and insertion of GLUT-4 into the cell membrane (4). However, LY-294002 prevents PI 3-kinase from producing phosphatidylinositol 3-phosphate products. It is likely, therefore, that the inability of the PI 3-kinase-ENaC complex to translocate to the membrane in LY-294002-treated cells results from the lack of formation of 3-phosphoinositides.

ENaC is an integral membrane protein that will be found as part of a membranous structure in the cytoplasmic storage form in the unstimulated cells. Our studies do not allow us to discern the nature of the membrane structure, although the ENaC in these studies does not appear to be part of large membrane vesicles. Whatever the nature of the membrane storage compartment, the change in lipid composition appears to alter its trafficking. Interestingly, the colocalization between the ENaC and PI 3-kinase, which appears to direct the storage form of ENaC to the lateral membrane, is lost as the channel enters the apical membrane.

Thus our data are most consistent with the hypothesis that activation of PI 3-kinase and the resulting formation of PIP3 regulate channel trafficking. If PI 3-kinase is important for the movement of ENaC into the apical cell membrane, then inhibiting the enzyme should result in a decrease in Na⁺ transport having a time course that reflects the half-life of ENaC in the cell membrane. The half-life of the channel in the membrane of A6 cells has been reported to be ~1 h (16). This process involves a dynamic balance between
channel exocytosis and endocytosis. Noise analysis was previously used to demonstrate that blocking PI 3-kinase with LY-294002 (50 μM) results in a decrease in basal Na⁺ transport to 23% of starting value within 1 h, an effect that is mirrored by a corresponding decrease in active channel number (13). The noise data were unable to distinguish between insulin-mediated activation of quiescent channels in the apical membrane and insertion of ENaC from an intracellular pool. The current results show that insertion from an intracellular pool is the most likely mechanism of insulin action and that this process is dependent on PI 3-kinase.

These results suggest an intriguing alternate route for transcellular signaling events in polarized epithelial cells. A signal, such as insulin receptor binding, that originates on the basolateral membrane can result in an apical membrane event by traveling around the cell membrane, perhaps using lipid intermediates to traverse the junctional complexes (5, 17). This hypothetical framework for signaling circumvents the difficulties inherent in proposing the intracellular movement of cytosolic signaling intermediates through relatively vast expanses of the intracellular milieu. However, proof for this novel mechanism will require live cell imaging with a variety of labeled intracellular probes.

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