Phosphatidylinositol 3,4,5-Trisphosphate Mediates Aldosterone Stimulation of Epithelial Sodium Channel (ENaC) and Interacts with γ-ENaC*

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Whole cell voltage clamp experiments were performed in a mouse cortical collecting duct principal cell line using patch pipettes back-filled with a solution containing phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 significantly increased amiloride-sensitive current in control cells but not in the cells prestimulated by aldosterone. Additionally, aldosterone stimulated amiloride-sensitive current in control cells, but not in the cells that expressed a PIP3-binding protein (Grp1-PH), which sequestered intracellular PIP3. PIP3 significantly increased amiloride-sensitive current in control cells, but not in the cells that expressed a PIP3-binding protein (Grp1-PH), which sequestered intracellular PIP3. 12 amino acids from the N-terminal tail (APGEKIKAKIKIK) of γ-epithelial sodium channel (γ-ENaC) were truncated by PCR-based mutagenesis (γT-ENaC). Whole cell and confocal microscopy experiments were conducted in Madin-Darby canine kidney cells co-expressing α- and β-ENaC only or with either γ-ENaC or γT-ENaC. The data demonstrated that the N-terminal tail truncation significantly decreased amiloride-sensitive current and that both the N-terminal tail truncation and LY-294002 (a PI3K inhibitor) prevented ENaC translocation to the plasma membrane. These data suggest that PIP3 mediates aldosterone-induced ENaC activity and trafficking and that the N-terminal tail of γ-ENaC is necessary for channel trafficking, probably channel gating as well. Additionally, we demonstrated a novel interaction between γ-ENaC and PIP3.

ENaC is a member of the ENaC/Deg superfamily of ion channels responsible for sodium transport across the apical membrane of a variety of epithelia including the colon, lung, and kidney (reviewed in Ref. 1). Since 1994, when ENaC was initially cloned from rat colon (2), the biophysical properties and molecular structure of ENaC have been extensively studied. Several lines of evidence suggest that ENaC is composed of three subunits, α, β, and γ, and that all three subunits are required to form a functional αβγ-ENaC channel complex (2–9).

Studying the mechanisms that regulate ENaC function is important because abnormal channel activity leads to several severe diseases. Constitutive activation of any component of ENaC subunits can cause Liddle’s syndrome, an autosomal dominant inherited disease that causes excessive sodium retention and hypertension. Conversely, loss of function mutations in α-, β-, or γ-ENaC causes pseudohypoaldosteronism type I, a hypertensive condition characterized by an inability to retain salt. These syndromes highlight the importance of normal ENaC activity in the kidney to maintain fluid and sodium homeostasis. The proper regulation of ENaC activity is also very important in the lung, because transgenic mice lacking functional channels die within 40 h of birth from fluid filled airways (10). Additionally, increases in intracellular Cl\textsuperscript{–} concentrations that secondarily lead to changes in ENaC activity play an important role in the pathophysiology of cystic fibrosis (11).

Anionic phospholipids, such as phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol 3,4,5-trisphosphate (PIP3), are normally located in the inner leaflet of the plasma membrane and are emerging as important regulators of ion transporters and channels. Although basal levels of both anionic phospholipids are generally very low, several models for the regulation of channels and transporters by PIP2 and PIP3 have been proposed. For example, all members of the inward rectifier potassium channel family (KATP, IRK, GIRK, and ROMK) are thought to be positively regulated by PIP2 interaction (reviewed in Ref. 12). Of these, the best characterized PIP3-binding domain is that of the KATP channel. PIP3 binds directly to the C terminus of the KATP channel, which contains multiple positively charged lysine and arginine residues and maintains an open conformation by preventing ATP binding (13–15). Classically, PIP3 is considered to be the lipid product generated when activated phosphoinositide 3-OH kinase (PI3K) phosphorylates PIP2 at the 3’ position and is the principle mediator of PI3K effects. Although little is known for its role in regulating the open state of channels, PIP3 does exhibit binding specificity and may be important in ion channel regulation by hormones and growth factors. It has recently been reported that PIP3 binds reversibly to regulators of G protein signaling molecules in cardiac cells to regulate K\textsuperscript{+} channel activity in response to changes in intracellular calcium levels. In a resting (low Ca\textsuperscript{2+}) state, the action of regulators of G protein signaling is thought to be allosterically inhibited by PIP2 (16). These studies demonstrate that anionic phospholipids can regulate various ion channels in many different systems and serve as possible analogous models for PIP3 regulation of ENaC activity in Na\textsuperscript{+} transporting epithelia.

Although the regulation of ENaC has been extensively studied, the specific regulation of ENaC by phosphoinositides remains largely unexplored. However, we have recently demonstrated that application of
PIPs, as well as PIP2, to the cytoplasmic surface of apical membranes of A6 cells and injected into Xenopus oocytes heterologously expressing ENaC prevented run down of ENaC activity and increased amiloride-sensitive channel activity in voltage clamp recordings (17, 18). Additionally, Tong et al. (19) demonstrated that PIP2 and PIP3 increased the open probability of reconstituted ENaC in excised patches of Chinese hamster ovary cells. Blazer-Yost and Nofziger have recently compared and contrasted the multiple effects of phosphoinositide lipids on ENaC in A6 and Chinese hamster ovary cells in a recent review (20).

In the present study, we examined the direct influence of PIP3 on ENaC activity in mpkCCDc14 clones, a mouse collecting duct principal cell line, which maintains aldosterone responsiveness and expresses functional ENaC endogenously (21, 22). We also truncated 12 amino acids from the N-terminal tail of γ-ENaC subunit, suggesting that full-length expression of this subunit is required for normal ENaC trafficking or stability at the plasma membrane. We also demonstrated a novel interaction between γ-ENaC and phospholipids, including PIP3 and PIP2.

MATERIALS AND METHODS

Cell Culture—The mouse cortical collecting duct principal cell line (mpkCCDc14) is often employed in the study of aldosterone-induced ENaC activity because of their specific responsive to physiological concentrations of mineralocorticoid hormone (21, 22). The mpkCCDc14 cells were incubated in a 1:1 mix of Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (Invitrogen) supplemented with 50 nM dexamethasone, 1 mM triiodothyronine, 20 mM HEPES, 2 mM l-glutamine, 0.1% penicillin/streptomycin, and 2% heat-inactivated fetal bovine serum. Madin-Darby canine kidney (MDCK) and Chinese hamster ovary cells (CHO) (obtained from ATCC, Manassas, VA) are also routinely used in investigating the regulation of sodium channel activity by exogenously expressing α-, β-, and γ-ENaC subunits (19, 23), because these cells do not express a significant amount of endogenous ENaC. MDCK cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum, and CHO cells were cultured in Hain’s F-12-Kaighn medium (Invitrogen) with 10% fetal bovine serum. All of the mammalian cell lines were maintained in plastic tissue culture flasks at 37 °C with 5% CO2 in air.

Generation of cDNA Constructs of Tagged ENaCs—Original plasmids containing cDNAs encoding the wild type α-, β-, and γ-ENaC in pSport vectors were provided by Dr. Bernard C. Rossier (University of Lausanne, Lausanne, Switzerland). A γ-ENaC-pDsRed2-N1 construct was created in three steps. First, a 1833-base pair fragment was excised from γ-ENaC-pSport using EcoRI and Apal restriction enzymes, which was then subsequently ligated into the corresponding cloning sites of pDsRed2-N1 vector (BD Biosciences, Palo Alto, CA). In the second step, a 242-base pair PCR fragment was synthesized using pCDNA3−γ-ENaC cDNA as a template, with a sense primer (TTGTGGGGCCCTGAGCAGA) corresponding to nucleotides 1814–1833 and an antisense primer (ACCGGTCGACACTGTCGACACT) corresponding to 2048–2032. This primer pair was chosen because unique Apal and Agel restriction sites are located within the primer sequences. The 242-base pair amplicon, lacking stop codons, was then QIAquick purified (Qiagen, Valencia, CA) and subsequently subcloned into the PGEM-TEasy vector (Promega, Madison, WI). In the third step, PGEM-TEasy containing the 237-base pair PCR product was digested with Apal/Agel and then ligated into the pDsRed2-γ-ENaC vector described above. In this way, we eliminated endogenous stop codons and cloned γ-ENaC in frame with pDsRed vector. The β-ENaC-pEYFP-N1 and α-ENaC-pECFP-N1 constructs were generated using similar strategies.

The γ-ENaC-pDsRed construct, encoding a protein in which the N-terminal tail of ENaC (APGEKIKAKIKK) is truncated, was created in three steps. First, a 36-nucleotide segment from the 5′ end of γ-ENaC was removed by EcoRI and BamHI enzyme digestion. Then a 393-PCR synthesized fragment was generated by using pCDNA3-γ-ENaC as a template and the primer pair 5′-ACCATGGCTCTCGGTTCGTA and 3′-GGACGCGATGGATCTGCT, to recreate the ATG start site and Kozak sequences in the truncated γ-ENaC-pDsRed construct. This was achieved by expressing the 393-base pair PCR fragment in pGEM-TEasy construct and subsequently cloning it in frame with pDsRed EcoRI and BamHI sites.

DNA Transfections—mpkCCDc14 cells were transfected with GFP-fused pleckstrin homology (PH) domains of either Grp1 or dynamin construct (obtained from Dr. Mark A. Lemmon, University of Pennsylvania School of Medicine, Philadelphia, PA); MDCK cells were transfected with fluorescently labeled α-, β-, and γ-ENaC constructs (described above), and CHO cells were transfected with either full-length γ-ENaC or the N-terminal tail truncated γ-ENaC construct or pDsRed vector alone. Each cell line was transfected using Lipofectamine Plus reagent (Invitrogen) in accordance with the manufacturer’s recommended protocol. Briefly, the cells were seeded at subconfluent densities 1 day before the transfection. DNA constructs were diluted with serum-free medium (1 μg DNA/50 μl medium), mixed with Plus reagent, and incubated at room temperature for 15 min. Then Lipofectamine reagent was diluted with serum-free medium (1 μl of Lipofectamine/25 μl of medium), mixed with the DNA/Plus solution, and incubated at room temperature for an additional 15 min. Finally, the transfection solution containing DNA, Plus reagent, and Lipofectamine reagent was applied to the cells and allowed to incubate for 4–6 h at 37 °C before the transfection solution was replaced with regular growth medium.

Patch Clamp Recording and Analysis—For patch clamp experiments, either MDCK cells or mpkCCDc14 were grown to confluent densities on permeable polyester membranes. The permeable support allowed patch pipette access to the apical membrane, as well as a physical separation of the apical and basolateral bath compartments. Immediately before use, the cells were thoroughly washed with NaCl bath solution (145 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES, at a pH of 7.4), transferred into the patch recording chamber mounted on a Nikon Eclipse TE200 inverted microscope, and then visualized with Hoffman modulation optics. The whole cell configuration was established with polished patch pipettes with a tip resistance of 2 MΩ. Only patches with a seal resistance above 10 GΩ were used for the experiments. Pipette solution contained 145 mM KCl, 5 mM NaCl, 50 mM free Ca2+ (after titration with 2 mM EGTA), 1 mM MgCl2, 2 mM K2-ATP, and 10 mM HEPES, at a pH of 7.2. A voltage step protocol from −120 mV to +40 mV (in 20-mV intervals) was used to monitor the current using an Axopatch 1-D (Axon Instruments, Union City, CA). The data were acquired using TL-1 acquisition hardware and analyzed with pClamp software (Axon Instruments). Patch clamp recordings were performed at room temperature.

Localization of PIP3 and ENaC Subunits with Laser Confocal Microscopy—According to techniques that have been previously established, the localization of PIP3 can be visualized after transfecting cells with a GFP-fused PH domain (24–27). Therefore, mpkCCDc14 cells were cultured on glass coverslips and then transiently transfected with the GFP-fused Grp1-PH construct to localize endogenous PIP3 in a collecting duct cell line. Fluorescently labeled ENaC subunits (γ-ENaC-pDsRed2-N1, β-ENaCpEYFP, and α-ENaCpECFP-N1) were also transfected into MDCK cells grown on permeable supports, as
FIGURE 1. PIP3 Stimulates ENaC in Control mpkCCDc14 Cells but Not in Aldosterone-treated mpkCCDc14 Cells—It is well known that blocking production of PIP3 by inhibiting PI3K blocks the effect of aldosterone on sodium transport in renal cells (29, 30). However, if an aldosterone-induced increase in PIP3 is the major cause of the initial hormone-induced increase in ENaC activity, then the addition of PIP3 to the cytosolic surface of renal cells should increase ENaC activity in the absence of aldosterone. Using the inside-out configuration, we recently demonstrated that PIP3 did not elevate but only maintained ENaC activity in aldosterone-treated A6 cells (17). We hypothesized that the stimulatory effect of PIP3 on ENaC activity may already be saturated in A6 cells that are continuously cultured in the presence of a high concentration of aldosterone. To test this hypothesis, in the present study we performed whole cell voltage clamp experiments in mpkCCDc14 cells, which do not require a high dose of aldosterone for growth and differentiation. Whole cell currents in response to a voltage step protocol (see Methods and Materials) were recorded from control mpkCCDc14 cells and aldosterone-treated mpkCCDc14 cells. The patch pipettes were back-filled with a solution containing 10 μM PIP3. Compared with the current immediately after forming the whole cell configuration, amiloride-sensitive current was significantly increased at 5 min (PIP3 had already diffused into the cells) in an aldosterone-free control cell (Fig. 1A) but was not increased or was increased to a lesser degree in an aldosterone-treated cell (Fig. 1B). Amiloride-sensitive currents at −100 mV in control cells were −0.30 ± 0.06 nA (control) and −1.30 ± 0.27
Regulation of ENaC by PIP₃

Aldosterone Elevates Membrane PIP₃ Concentration via PI3K—As we have described above, the Grp1-PH domain binds strongly and specifically to PIP₃ and is therefore commonly employed in the study of PIP₃ in vivo. By fusing Grp1-PH to GFP, we were able to localize PIP₃ expression in mpkCCDc14 cells. In the absence of serum and aldosterone, we expect GFP-Grp1-PH expression to be evenly distributed across the cytoplasm with low fluorescence intensity, because PIP₃ levels under resting conditions are very low. However, aldosterone-induced activation of PI3K should enhance PIP₃ levels at the plasma membrane.

To test our hypothesis that aldosterone elevates membrane PIP₃ concentrations via PI3K, we first transfected mpkCCDc14 cells with the GFP-fused Grp1-PH domain (Fig. 3). The cells that were deprived of serum and hormone did not contain significant amounts of PIP₃. In this basal state, the expressed GFP-fused Grp1-PH domain was distributed with an even intensity across the whole cytoplasm of mpkCCDc14 cells as expected (left panel). Cells treated with 1 μM aldosterone for 30 min (middle panel) displayed predominant GFP-fluorescence intensity at the plasma membrane. This effect was prevented by pretreating the cells with 5 μM LY294002, a specific PI3K inhibitor (right panel), indicating that aldosterone can elevate the concentration of PIP₃ in the plasma membrane (where functional ENaC resides) by stimulating PI3K. These data suggest that PIP₃ is an important regulator of aldosterone-induced sodium channel activity.

Truncation of γ-ENaC N-terminal Tail Decreases Amiloride-sensitive Sodium Current—The N termini of ENaC subunits are very important in normal ENaC function. It has been shown that deletion of positively charged motifs in the cytoplasmic N termini of β- (Δ2–49) and γ-ENaC (Δ2–53) dramatically reduces ENaC activity (34). We determined whether removal of 12 amino acids (Δ2–13), which include several conserved lysine residues (shown in Fig. 4D) from the N-terminal tail of γ-ENaC, would lead to a reduction in amiloride-sensitive current and alter subunit translocation in MDCK cells.

The N-terminal truncated form of γ-ENaC was subcloned into pDsRed2 vector (γ-ENaC). ENaC was then reconstituted in MDCK cells by co-transfection of either full-length αβγ-ENaC or αβγ-γ-ENaC. Transfected cells were pretreated with 1 μM aldosterone for 2 h before obtaining the whole cell configuration. The data demonstrated that the current in cells expressing full-length αβγ-ENaC (Fig. 4A) was 10 times higher than cells expressing αβγ-γ-ENaC (Fig. 4B). The mean amiloride-sensitive current at −100 mV was −2.25 ± 0.39 nA (n = 5) in αβγ-
Regulation of ENaC by PIP₃

FIGURE 3. Aldosterone elevates membrane concentrations of PIP₃ in mpkCCDc14 cells transfected with GFP-fused Grp1-PH domain to indirectly map cellular PIP₃, because of its high affinity binding to PIP₃. Experimental were performed under control conditions (left panel) and after pretreatment with either 1 µM aldosterone alone (middle panel) or 1 µM aldosterone in the presence of 5 mM LY294002 (right panel). The data represent three independent experiments.

FIGURE 4. Reduction of amiloride-sensitive current in MDCK expressing αβγ-ENaC. Representative whole cell current was recorded from MDCK cells transfected with either αβγ-ENaC (A) or αβγ-ENaC (B) and cultured in the presence 1 µM aldosterone. The current was recorded under control conditions (left traces), after perfusion of cells with 2 µM amiloride (middle traces), and after washing the bath (right traces). C, mean amiloride-sensitive current at -100 mV in MDCK cells transfected with either αβγ-ENaC or αβγ-ENaC and in the presence 1 µM aldosterone. D, truncated amino acid sequence of rat γ-ENaC subunit at the N-terminal end.

FIGURE 5. Abolishment of aldosterone-induced ENaC trafficking to the plasma membrane in MDCK cells expressing αβγ-ENaC. Wild type α- and β-ENaC were co-expressed with either wild type γ-ENaC (A–C) or N-terminal tail-truncated γ-ENaC (D) in MDCK cells. α-, β-, or γ-ENaC was tagged with fluorescent proteins CFP, YFP, and pDsRed, respectively. Confocal microscopy experiments were performed in serum- and hormone-deprived control cells (A) and cells treated with either 1 µM aldosterone alone (B and D) or 1 µM aldosterone in the presence of 5 mM LY294002 (C) for 2 h. The data represent five independent experiments showing similar results.

ENaC-transfected MDCK cells, which was 10 times higher than in cells expressing αβγ-ENaC (−0.24 ± 0.064 nA, n = 5) (p < 0.0001).

Truncation of γ-ENaC N-terminal Tail and Inhibition of PI3K Impede ENaC Translocation to the Plasma Membrane—Because α-, β-, γ-, and γ-ENaC subunits were cloned into pEFP, pEFY, pDsRed, and pDsRed vector, respectively, we were able to perform confocal microscopy experiments to determine the effect of the N-terminal tail truncation and γ-ENaC deletion on sodium channel translocation to the plasma membrane in MDCK cells. The localization of α-ENaC (blue), β-ENaC (yellow), or γ-ENaC (red) subunit in the cells is shown separately in the first three panels of Fig. 5 and is then superimposed in the last panel. Compared with that under control conditions (Fig. 5A), wild type αβγ-ENaC subunits all translocated to the plasma membrane at 2 h after 1 µM aldosterone treatment at 37 °C (Fig. 5B). Aldosterone-induced trafficking of wild type ENaC subunits to the plasma membrane was abolished by 5 mM LY294002 (Fig. 5C), strongly suggesting that PI3K-generated lipids (such as PIP₃) provide a recruitment mechanism for ENaC to the apical membrane. Importantly, expression of an N-terminal truncated γ-ENaC (γ₋ENaC) with full-length α- and β-ENaC subunits prevented ENaC trafficking to the plasma membrane (Fig. 5D) and is consistent with our finding that αβγ-ENaC expression in MDCK cells leads to decreased amiloride-sensitive current.

We performed additional experiments in which only α- and β-ENaC subunits were expressed in MDCK cells. Fig. 6 shows that the α- and β-ENaC subunits can be efficiently expressed in the absence of γ-subunit. Using the same excitation wavelengths and gain settings in confocal analysis, it appears that α- and β-ENaC expression levels are similar to the levels reached by transfecting all three ENaC subunits in MDCK cells, shown in Fig. 5. However, no detectable amiloride-sensitive current was observed in α,β-ENaC only, co-transfected cells (data not shown). Furthermore, Fig. 6 shows that the α and β subunits do not effectively traffic to the plasma membrane in response to 1 µM aldosterone treatment in the absence of γ-ENaC expression. Although ENaC trafficking is greatly limited in these cells, there is still some membrane localization of the α- and β-subunits. Our findings show that complete, full-length expression of the α-, β-, and γ-ENaC subunits are requisite for the formation as well as effective translocation of functional sodium transporting channels at the apical membrane of kidney cells.
Regulation of ENaC by PIP₃

Phospholipid Binding Specificity of γ-ENaC—Because our data show that anionic phospholipids can mediate ENaC activity, we next tested the ability of γ-ENaC to bind to various phospholipids using a protein-lipid overlay method. Phospholipids, including phosphoinositides, were spotted onto a membrane (Echelon Bioscience Inc.) and incubated with either cell lysate from αβγ-ENaC- or αβγ₁-ENaC-transfected CHO cells. The membranes were then washed and immunoblotted using anti-DsRed antibody to detect γ-ENaC-pDsRed2-N1 binding to the membrane, via direct interactions with the lipids. As shown in Fig. 7A, both γ₁-ENaC and γ-ENaC interacted with 100 pmol of P(3,4)P₂, P(3,5)P₂, P(4,5)P₂, P(3,4,5)P₃, phosphatic acid, phosphatidylycerine, PI, P(3)P, P(4)P, and P(5)P. The ENaC subunits did not bind to sphingosine-1-phosphate, lysophosphatidic acid, lysophosphocholine, phosphatidylethanolamine, phosphatidylcholine, nor the control in which no lipid was spotted onto the membrane (position 8 on the strips). As an additional control, we also demonstrated that DsRed protein alone did not bind nonspecifically to membrane that had only been incubated with protein lysate from pDsRed-transfected CHO cells.

Fig. 7B (left panel) also confirms effective γ- and γ₁-ENaC-pDsRed2-N1 expression in CHO cells and that these ENaC subunits can pull down with 1.5 pmol of PIP₃ bound beads. We show in the right panel, as a negative control, that the DsRed label alone does not contribute to PIP₃ protein pull-down. Together, these data show that both the full-length and N-terminal tail truncated forms of γ-ENaC bind to phospholipids spotted on a nitrocellulose membrane and can pull-down with PIP₃ immobilized on beads.

DISCUSSION

We previously demonstrated that anionic phospholipids including PIP₃ maintained ENaC activity in inside-out patches excised from aldo- 
stosterone-conditioned A6 cells (17). Although results from this previous publication convincingly showed that PIP₃ increases the likelihood that ENaC would be open, these single channel studies could not discern whether an increase in the number of channels trafficked to the apical membrane could also be responsible for maintaining ENaC activity in PIP₃-treated cells. Our current study coupled with our previous observations suggest that PIP₃ serves as an effective regulator of ENaC trafficking (from the cytoplasmic pool to the surface membrane), promotes an open state of the channel, or maintains channel stability through direct interactions with the ENaC subunit at the plasma membrane.

Using the mouse mpkCCD cell line, which does not require aldosterone for growth and differentiation, we demonstrated that PIP₃ stimulated ENaC activity in the absence of aldosterone but could not further increase ENaC activity in the presence of aldosterone. We also showed that aldosterone elevated the concentration of PIP₃ in the plasma membrane and that sequestering PIP₃ with Grp1-PH domain prevented aldosterone activation of ENaC. Although the α- and β-subunits were expressed at high levels in MDCK cells, αβ-ENaC complexes could not completely traffic to the plasma membrane in the absence of γ-ENaC expression, because we could measure no amiloride-sensitive current (data not shown). Truncation of lysine-rich residues in the N-terminal end of γ-ENaC similarly inhibited aldosterone-induced increases in current and prevented appropriate channel translocation to the plasma membrane after aldosterone treatment. We originally thought that this might be due to a failure of γ₁ to bind PIP₃, but our lipid overlay assays revealed that the γ₁-ENaC binds to phospholipids, including PIP₃. It appears that the binding ability of the N-terminally truncated form of γ-ENaC was slightly higher than that of wild type. Therefore, it is possible that the N-terminal tail is required for channel gating or trafficking for reasons other than lipid binding. A report just came out suggesting that the region immediately following the second transmembrane span- ning domain of γ-ENaC acts as part of a functional PIP₂-binding site (35). We are currently investigating additional arginine- and lysine-rich domains in ENaC subunits, which may directly interact with anionic phospholipids, as hypothesized in our recent review article (36).

However, our current model for the regulation of ENaC by anionic phospholipids does not exclude a role for the serum and glucocorticoid-inducible kinase (SGK1), an immediate aldosterone induced kinase that increases the activity of ENaC (37–43). The upstream regulators of SGK1 enzyme activity are 3-phosphoinositide-dependent kinase-1 and -2 (PDK1 and PDK2, respectively); thus SGK1 is also inhibited by PI3K inhibitors such as LY294002 and is dependent upon PIP₃ for complete activation (44, 45). PIP₃ may enhance ENaC function by associating with
SGK1 and recruit this kinase to the inner leaflet of the plasma membrane. Once at the appropriate site of PDK1 and PDK2 activation, SGK1 could then inhibit ubiquitin ligase Nedd4–2 activity (as we currently understand it to). Because normal ENaC function is so important in maintaining fluid and ion homeostasis, it makes sense that tight epithelial cells would utilize multiple pathways to ensure net Na$^+$ re-uptake.

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