Phosphatidylinositol 4,5-bisphosphate directly interacts with the β and γ subunits of the sodium channel ENaC

Crystal R. Archer1,2,* 1, Benjamin T. Enslow2, Chase M. Carver1, and James D. Stockand1

From the 1Department of Cellular and Integrative Physiology and 2Division of Nephrology, Department of Medicine, University of Texas Health Science Center, San Antonio, Texas, United States

Edited by Roger J. Colbran

The plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) regulates the activity of diverse ion channels to include the epithelial Na⁺ channel ENaC. Whether PIP2 regulation of ENaC is due to a direct phospholipid-protein interaction, remains obscure. To date, possible interaction of PIP2 with ENaC primarily has been tested indirectly through assays of channel function. A fragment-based biochemical analysis approach is used here to directly quantify possible PIP2-ENaC interactions. We find using the CIBN-CRY2 optogenetic dimerization system that the phosphoryl group positioned at carbon 5 of PIP2 is necessary for interaction with ENaC. Previous studies have implicated conserved basic residues in the cytosolic portions of β- and γ-ENaC subunits as being important for PIP2-ENaC interactions. To test this, we used synthetic peptides of these regions of β- and γ-ENaC. Steady-state intrinsic fluorescence spectroscopy demonstrated that phosphoinositides change the local conformation of the N terminus of β-ENaC, and two sites of γ-ENaC adjacent to the plasma membrane, suggesting direct interactions of PIP2 with these three regions. Microscale thermophoresis elaborated PIP2 interactions with the N termini of β- (Kd ~ 5.2 μM) and γ-ENaC (Kd ~ 13 μM). A weaker interaction site within the carboxyl terminus of γ-ENaC (Kd ~ 800 μM) was also observed. These results support that PIP2 regulates ENaC activity by directly interacting with at least three distinct regions within the cytoplasmic domains of the channel that contain conserved basic residues. These interactions are probably electrostatic in nature, and are likely to bear a key structural role in support of channel activity.

The activity of the amiloride-sensitive, epithelial Na⁺ channel (ENaC) is limiting for sodium transport across many absorptive epithelial tissues to include those lining the renal distal nephron, the lungs and colon. ENaC is expressed in the apical plasma membranes of polarized epithelial cells that form these tissues (1). This function and location in the kidney makes ENaC the final arbiter of renal sodium excretion. As such, the activity of ENaC plays a key role in the normal control of blood pressure by affecting renal sodium excretion (2). Mutation of ENaC, consequently, causes inheritable forms of hyper- and hypotension (1, 3). Moreover, because of its function and expression in epithelial cells of the airways and lungs, ENaC activity is critical to normal hydration of airway mucus and fluid clearance from alveolar spaces (4). ENaC dysfunction in these latter tissues, thus, can contribute to the pathology of cystic fibrosis and respiratory distress (5).

The bulk of the protein structure of ENaC, as elaborated by cryo-EM, is known (6). Similar to the related acid-sensing ion channel 1 (ASIC1), the first member of the ENaC/Deg ion channel family to be crystallized (7), ENaC is a trimeric ion channel. Specifically, ENaC is a heterotrimer consisting of related α, β, and γ subunits, which are encoded by distinct genes (6). The overall structure of ENaC/Deg ion channels, as exemplified by ENaC and ASIC1, is similar to that of the unrelated ionotropic P2X receptors (8). Each ENaC subunit contains two transmembrane domains, a large extracellular domain, and relatively short cytosolic amino and carboxyl termini. ENaC subunits fit together to form a chalice-like structure with an internal pore that spans the plasma membrane. The bulk of the channel is extracellular and formed of large globular domains. The channel pore is formed by the helical second transmembrane domains of each subunit as they run perpendicular to the plasma membrane. Similar to that for crystallization of ASIC1, the cytoplasmic regions of ENaC were truncated to facilitate the resolution of structure. Thus, the structure of the intracellular domains of ENaC are currently obscure. Strong evidence, although, supports that these cytoplasmic domains are key cell signaling targets during the normal regulation of channel activity. Signaling to cytoplasmic domains within the ENaC influence channel expression, turnover, and gating (9–12). A physiologically important regulator of ENaC activity that is thought to influence the channel through interactions with intracellular domains is the plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2) (2, 13, 14).

PIP2 effects on ion channels were first reported in the early 1990’s where PIP2 was shown to stimulate Ca2⁺ current through ionotropic ryanodine receptors, and prevent rundown of KATP channels in excised membrane patches (15, 16). The dependence on local PIP2 of the inward rectifying K⁺ channel, Kir, for normal channel activity was clearly demonstrated a few years later by Hilgemann et al. (17, 18). In these studies, depletion of PIP2 reduced the activity of Kir whereas, exogenous PIP2 increased channel activity. PIP2 was found to directly interact with critical cationic residues within cytoplasmic domains of Kir (18). Since these pioneering studies, PIP2 has been shown to be a key regulator of many distinct ion channels to include voltage-gated K⁺ channels, 2-pore K⁺ channels, P2X receptors,
TRP channels, and N-type and P/Q-type voltage gated Ca\(^{2+}\) channels (19, 20). Many of these channels contain clusters of cationic residues within cytoplasmic domains critical to their PIP\(_2\) dependence. These cationic sites resemble those of the pleckstrin homology and ENTH PIP\(_2\)-binding domains (19), and are suspected to bind the exposed phosphoryl group of PIP\(_2\), commonly referred to as the “PIP\(_2\) headgroup.” Electrostatic PIP\(_2\)-ion channel interactions have been directly observed in the cryo-EM and crystal structures of TRPM8, TMEM16F, K\(_{\alpha}2.2\), and K\(_{\alpha}3.2\) (22–25). However, the precise mechanism by which PIP\(_2\) regulates diverse ion channels is still debated. Although PIP\(_2\) is reported to inactivate some channels, including hEAG1 (26) and TRPL (27), most channels sensitive to this phospholipid are activated by it (19). To date, ENaC is the only Na\(^{+}\)-selective ion channel reported to be activated by PIP\(_2\) (14, 28).

The effects of PIP\(_2\) on ENaC have primarily been characterized in electrophysiology studies of channel function. For instance, manipulation of phospholipid levels in excised, inside-out cell patches containing heterologously expressed ENaC revealed that PIP\(_2\) stabilizes ENaC currents and prevents channel rundown (29). Moreover, stimulation of G protein–coupled receptors, which are coupled via Gi to PLC, facilitates hydrolysis of PIP\(_2\) leading to a decrease in ENaC activity in native renal tubules (20). Co-immunoprecipitation studies combined with site-directed mutagenesis are consistent with, but not definitive of, PIP\(_2\) directly interacting with clusters of basic residues within the cytoplasmic regions of the \(\beta\)- and \(\gamma\)-, but not \(\alpha\)-ENaC subunits (14, 30). More recent evidence suggests that PIP\(_2\) upon interacting with the channel may increase the helical content of the N terminus of \(\gamma\)-ENaC (31). Although substantial proof exists for ENaC’s dependence on PIP\(_2\) and probable interaction of this phospholipid with the channel, specific details about the strength and exact channel domains involved in such interactions have remained obscure.

The current study addresses this deficiency by directly quantifying PIP\(_2\)-ENaC interactions using a quantitative biophysical approach capable of elaborating the binding affinities of such interactions as well as precisely defining the domains within the channel involved in these interactions. We hypothesized that the headgroups of PIP\(_2\) directly interact with multiple clusters of cationic residues conserved within the cytoplasmic termini of \(\beta\)- and \(\gamma\)-ENaC subunits. Optogenetic manipulation of PIP\(_2\) demonstrated that the PIP\(_2\) headgroup is necessary for effects on ENaC. Steady-state intrinsic fluorescence spectroscopy and microscale thermophoresis in conjunction with mutationesis identified three distinct clusters of cationic residues conserved in the cytoplasmic termini of \(\beta\)- and \(\gamma\)-ENaC as being necessary for channel interactions with PIP\(_2\). These three PIP\(_2\)-binding sites within ENaC interact with the phospholipid with low to moderate affinity in the micromolar range.

Results

Blue light-recruitment of 5-ptase reduces membrane PIP\(_2\) levels

We hypothesized, as others have before (14, 30), that phosphoryl groups at carbons 4 and 5 (C4 and C5) of PIP\(_2\), illustrated in Fig. S1, are necessary for PIP\(_2\)-ENaC interactions. Targeted removal of the C5 phosphoryl group of PIP\(_2\) was accomplished using the recently developed CIBN-CRY2–5-phosphataseOCRL optogenetics system. This system has been described previously in the study of PIP\(_2\) regulation of KCNQ K\(^{+}\) channels (32). In brief, proteins in this system have been engineered such that the CAAX-fused CIBN is localized to the plasma membrane, and mCherry-CRY2 fused to the 5-phosphatase OCRL (mCh-CRY2–5-ptase) is expressed in the cytoplasm. CRY2 dimerizes with CIBN upon exposure to blue light. Upon blue light illumination (BLI), the 5-ptase translocates to the membrane where it dephosphorylates PIP\(_2\) at C5.

To confirm the functionality of the CIBN/CRY2–5-ptase dimerization system on PIP\(_2\) in our hands, we followed the localization pattern of the cellular PIP\(_2\) reporter YFP-Tubby. This reporter selectively binds PIP\(_2\) over inositol triphosphate (IP\(_3\)) (33, 34). Blue light activation of CIBN/mCh-CRY2–5-ptase was achieved using brief global illumination through a standard CFP fluorescence filter. Diffuse, cytoplasmic YFP-Tubby (green) was consistently observed in cells exposed to BLI that also expressed mCh-CRY2–5-ptase (red) (Fig. 1A). In contrast, even with BLI, cells expressing only YFP-Tubby displayed stronger membrane localization, consistent with brighter fluorescence along the edges of the cells. This is indicated by the white arrows in the merged images of Fig. 1A. This change in localization is consistent with Tubby being recruited to the membrane via association with PIP\(_2\) but translocating to the cytoplasm when PIP\(_2\) is depleted. TIRF imaging was used to more specifically monitor the effects of BLI-induced recruitment of mCh-CRY2–5-ptase to the membrane on the location of YFP-Tubby. This strategy, combined with finding cells that express mCherry with 561 nm illumination, and imaging Tubby at 514 nm, enabled us to selectively activate cells independent of each other. Although the mCh-CRY2–5-ptase changes tended to be subtle following BLI, YFP-Tubby membrane fluorescence immediately dropped to 58 ± 7% of starting levels, suggesting a significant and intact phosphoinositide at the membrane (Fig. 1, B and C). Tubby levels recovered in 5–10 min to 78 ± 15% of starting levels. This experiment is consistent with the CIBN/CRY2–5-ptase dimerization system causing a rapid and significant reduction in membrane PIP\(_2\) upon BLI.

Na\(^{+}\) flux through ENaC depends on the C5 phosphoryl group of PIP\(_2\)

The activity of ENaC in excised, inside-out patches is influenced by the levels of PIP\(_2\) in the plasma membrane (14, 29). To further elaborate PIP\(_2\) effects on ENaC, we quantified changes in Na\(^{+}\) flux through human ENaC (hENaC) before and after the targeted removal of the C5 phosphoryl group of PIP\(_2\) using the CIBN/CRY2–5-ptase dimerization system. CoroNa Green fluorescent sodium-selective indicator was used to monitor changes in intracellular Na\(^{+}\) levels in HEK 293 cells transfected with mCh-CRY2–5-ptase and CIBN-CAAX, with and without hENaC before and after BLI. We recorded changes in intracellular Na\(^{+}\) levels as a function of normalized change of CoroNa Green fluorescence using a method similar to that used previously to detect intracellular Na\(^{+}\) changes in cortical neurons (35). The sensitivity of CoroNa Green to Na\(^{+}\) changes affected
by ENaC activity was first tested using the selective blocker amiloride (Fig. S2, black bars). Basal Na\(^+\) levels in HEK 293 cells were initially estimated in the presence of 10 \(\mu M\) amiloride. Removal of amiloride by perfusion of PBS resulted in a significant increase of Corona Green fluorescence by \(\sim 68\%\), consistent with an increase of intracellular Na\(^+\) through ENaC upon removal of the channel blocker. Replication of 10 \(\mu M\) amiloride significantly decreased Corona Green fluorescence \(\sim 63\%\) to levels near starting values in amiloride. In contrast, removing amiloride had no effect on Corona Green fluorescence in control cells not expressing ENaC (Fig. S2, purple bars). These results support the use of Corona Green for detecting changes in intracellular Na\(^+\) levels via ENaC. Thus, a reduction in the magnitude of green fluorescence indicates a decrease in intracellular [Na\(^+\)] consistent with a decrease in Na\(^+\) influx upon inhibition of ENaC.

We next tested the effects of depleting PIP\(_2\) on ENaC activity using this system. Because of the quick depletion of PIP\(_2\) and its subsequent recovery observed in Fig. 1, we imaged CoroNa at 488 nm, with an additional flash at 5 min to retain membrane-localized mCh-CRY2–5-ptase for the duration of the experiment. The representative wide-field fluorescence micrographs in Fig. 2A show that prior to BLI, the mCh-CRY2–5-ptase fluorescence is diffuse throughout the cell (left). Following BLI, mCh-CRY2–5-ptase fluorescence increases at the plasma membrane (middle). Recruitment of mCh-CRY2–5-ptase to the membrane was quantified by subtracting mCherry fluorescence across each cell prior to BLI from that 10 min after BLI shown in the differential fluorescence micrographs (right). Intensity plots for regions of interest across each cell document that significant peak-BLI-sensitive changes in mCherry fluorescence occur at the edges of these cells consistent with the plasma membrane (Fig. 2, B and C). In contrast, there was no significant difference in the overall fluorescence of the cytoplasmic (middle) region of each cell (Fig. 2C). The summary graph in Fig. 2D shows that changes in membrane mCh-CRY2–5-ptase following BLI occur independent of co-expression of hENaC. However, as shown in the representative fluorescence micrographs in Fig. 2E and summarized in Fig. 2F, the magnitude of CoroNa Green fluorescence decreases as a function of hENaC expression and translocation of mCh-CRY2–5-ptase to the plasma membrane following BLI. In cells expressing hENaC, normalized (to starting values) CoroNa Green fluorescence was significantly decreased by 25.2 \(\pm\) 4.6\% 10 min after BLI. In contrast, CoroNa Green fluorescence slightly, but not significantly, increased by 4.5 \(\pm\) 8.5\% 10 min after BLI in control cells that were not transfected with hENaC, suggesting that this decrease was not due to leakage or bleaching of CoroNa Green. These results are consistent with the C5 phosphoryl headgroup of membrane PIP\(_2\) being necessary for normal ENaC function.

**Cationic residues within the cytosolic termini of \(\beta\)- and \(\gamma\)-ENaC potentially cluster to form PIP\(_2\)-binding sites**

Based upon our previous findings that clusters of cationic residues within the cytoplasmic termini of \(\beta\)- and \(\gamma\)-mENaC (mouse ENaC) are involved in the regulation of the channel by PIP\(_2\) (14), we designed synthetic peptides corresponding to these regions of hENaC. Table S1 shows the peptide sequences used for this study and their corresponding mutants studied in
our prior work (14). The positions of these peptides within \( \beta \)- and \( \gamma \)-ENaC subunits are shown in the illustrations (A) and sequence alignments (B and C) of Fig. 3. Notably, these domains within ENaC are highly conserved. In a recent study (31), it was suggested that PIP\(_2\) promoted assumption of a helical structure by the N terminus of \( \gamma \)-ENaC. JPred4 online software was used to identify potential regions of helical content within the amino and carboxyl termini of \( \beta \)- and \( \gamma \)-ENaC, indicated by a series of red “H” below the sequence alignments in Fig. 3. The candidate peptides studied here predicted to have the greatest helical content are \( \beta N1 \), \( \gamma N1 \), and \( \gamma C \).

Helical wheel diagrams were generated, as shown in Fig. 4, to evaluate the potential distribution of the positively charged residues of these peptides if they were to form helices. Clustering of cationic residues secondary to protein folding may increase the likelihood for electrostatic interactions with the negatively charged phospholipid groups of PIP\(_2\). The \( \beta N1 \) helix model displays a cluster of 7 basic residues to one side of the putative helix. Smaller clusters are observed within \( \gamma N1 \), \( \gamma N2 \), and \( \beta N2 \). \( \gamma C \) and \( \beta C \) each present at least one doublet of basic residues, which may also serve as phosphoinositide interaction sites. These bioinformatics data are consistent with helical structures within the cytoplasmic domains of ENaC subunits, should they occur, forming electropositive regions that would likely be receptive to interaction with the negative phosphoryl headgroups of PIP\(_2\).

**Phosphoinositides cause a change in emission spectra of \( \beta N1 \), \( \gamma N2 \), and \( \gamma C \) ENaC peptides**

Ponchynyuk et al. (14) found that mutations and deletions within the extreme N termini of \( \beta \)- and \( \gamma \)-mENaC, \( \beta N D \) (corresponding to the \( \beta N1 \) region in this study), and \( \gamma N S \) (\( \gamma N1 \) in the current study), abrogated ENaC responses to depletion of PIP\(_2\), suggesting these regions are important for PIP\(_2\)-sensitive responses. Likewise, channels with mutations in the carboxyl termini adjacent to the membrane, \( \beta 2 D \) (\( \beta C \) in the current study) and \( \gamma 2 S / D \) (\( \gamma C \) in the current study), did not respond to phosphatidylinositol 3-kinase and increases of the related phospholipid, phosphatidylinositol 3,4,5-trisphosphate (PIP\(_{3}\)) suggesting these sites were perhaps also important for responses to changes in phospholipid levels. ENaC containing mutants in the N termini adjacent to the membrane, \( \gamma 1 D / S \) (\( \gamma N2 \) in the current study) and \( \beta 1 D / S \) (\( \beta N2 \) in the current study) did not produce detectable Na\(^{+}\) currents in this earlier

---

**Figure 2. Depletion of membrane PIP\(_2\) slows the influx of Na\(^{+}\) into HEK 293 cells in the presence but not absence of hENaC.** A, HEK 293 cells transfected with human ENaC, mCh-CRY2–5-ptase and CIBN-CAAX before (left) and 10 min after (middle) BLI pulses. Representative micrographs are shown (right) for hENaC expressing cells for which fluorescence from mCh-CRY2–5-ptase before BLI was subtracted and 10 min after BLI. Note that panels B and E correspond to the representative cells in A. B, summary graphs comparing the relative fluorescence of mCh-CRY2–5-ptase across the indicated cell to the left, marked by a line in the micrographs of subtracted CRY2 levels shown in A. The fluorescence across each cell before BLI is shown in purple, and 10 min after shown in cyan. Changes at the plasma membrane are noted by peaks and indicated by black arrows. C, summary of changes of mCh-CRY2–5-ptase membrane fluorescence at the right and left edge of each cell compared with the middle of the cell before (purple) and 10 min after (cyan) BLI. D, summary graph of the relative change in membrane mCh-CRY2–5-ptase fluorescence 10 min after BLI, as compared with before, of cells with and without ENaC. E, representative micrographs of CoroNa Green fluorescence before (left) and 10 min after (right) BLI, corresponding to the cells shown in A. F, summary graph comparing intracellular CoroNa Green levels before and 10 min after BLI in HEK 293 cells not transfected with hENaC (left) and in those expressing the channel (right). Data are from \( n = 10–11 \) cells for each group; **, \( p < 0.005 \), n.s., not significant.
Figure 3. Putative PIP₂-binding sites within ENaC subunits. A, cartoons demarking putative PIP₂-binding sites in the cytosolic termini of β- and γ-ENaC subunits. B and C, alignment of the cytosolic termini of the β- and γ-ENaC subunits from human and mouse: * indicates conserved residues. Blue and green boxes indicate the human sequences used as synthetic peptides in these studies. Also shown below each alignment are the results of a JPred4/Jnet analysis indicating sites of predicted helical structure. Red H and orange E indicate that these domains within ENaC subunits have a high and moderate probability, respectively, of forming an α helix.

Figure 4. Clustering of cationic residues by helical formation of hENaC cytoplasmic termini. Helical wheel projections of the sequences for each peptide corresponding to the putative PIP₂ sites in β- and γ-hENaC subunits. The peptide label is shown inside each wheel. Residues with basic side chains, H, K, and R, are circled with thick black lines. Hydrophobic residues marked with blue squares, and acidic residues and hydroxyl- and nitrogen-containing residues marked with red diamonds. Helical projections were made using the online tool EMBoss Pepwheel.
work, and consequently, could not be tested for phospholipid sensitivity. It has been reported previously that this region of H9253-ENaC is also important for interacting with PIP2 (31). Here we studied the direct biochemical interactions of peptides corresponding to these regions of ENaC with soluble analogs of PIP2 and PIP3. We first examined the steady-state intrinsic fluorescence (SSIF) of each ENaC peptide in the absence and presence of diC4-PIP2 and diC4-PIP3. The latter are synthetic phospholipids that have shortened fatty acyl tails to improve their solubility in physiological salt solutions. The simplified molecular structures shown in Fig. S1 highlight the positions of critical functional groups in these phospholipids. The PIP2 and PIP3 analogs used for this study do not absorb at the same wavelengths (250 and 280 nm) as aromatic amino acids (Fig. S3, A and B). This fact enabled us to evaluate PIP2- and PIP3-dependent changes in the emission spectra of ENaC peptides as a faithful readout for phosphoinositide binding.

Fluorescence emission spectra were recorded for each ENaC peptide (0.25 M) before and after addition of PIP2 (Fig. 5) and PIP3 (Fig. 6) with excitation at 250 and 280 nm. The prior wavelength documents changes in the local environment following emissions from regions containing tyrosine (Tyr) residues and the latter from those containing tryptophan (Trp) residues (36). Although 250 nm typically is also used to follow local changes in structure in regions that include phenylalanine (Phe) (36, 37), none of the ENaC peptides studied here contained Phe. Consequently, changes observed at 250 nm reflect local changes around Tyr. Phosphoinositides were added to each peptide in excess (10 M) and incubated at room temperature at least 1 min prior to recording to facilitate binding.

Addition of diC4-PIP2 (Fig. 5) caused peak changes of 9–16% for N1, N2, and C after excitation at 250 nm, and 6–14% at 280 nm. As documented in Fig. 5, D and E, these peak changes were significantly greater in WT peptides as compared with peptides that contained mutations of their basic residues. Because charge-neutralized mutants were insoluble, all mutations used in these studies consisted of anionic glutamate in the place of cationic residues, resulting in charge reversal (Table S1, blue text). Mutant peptides had peak changes in response to PIP2 of only 1–3%. Addition of diC4-PIP3 (Fig. 6) also caused greater changes in emissions from N1, N2, and C peptides (7–14%) as compared with their mutants; however, only N1 and N2 excited at 280 nm had changes significantly greater than their corresponding mutants. No significant difference was observed in responses between C, N2, and N1 and mutant peptides for all PIP2 data sets (2-way ANOVA; interaction: F(5,24) = 0.7758, p = 0.5767; Fwavelength(1,24) = 1.188, p = 0.2866; Fwt vs mutant(5,24) = 1.282, p = 0.3041. Data are presented as mean ± S.D. from 3 replicates.

Figure 5. PIP2 effects on the steady-state intrinsic fluorescence of hENaC cytosolic fragments. Emission spectra of hENaC peptides excited at 250 (left panel) and 280 nm (right panel) in the absence (black line) and presence (cyan line) of diC4-PIP2. Emissions spectra for βN1, βN2, and βC peptides in A; γN1, γN2, and γC peptides in B; and mutant peptides in C. Quantification of the changes in peak emissions with excitation at 250 (D) and 280 nm (E). Summary results for WT and mutant peptides are shown as black and white histograms, respectively. Significant differences between the WT and mutants were noted for βN1, γN2, and γC (multiple t tests; *, p < 0.05; **, p < 0.005). In contrast, no significant difference was noted between βN2, βC, γN1, and mutant peptides for all PIP2 data sets (2-way ANOVA; interaction: F(5,24) = 0.7758, p = 0.5767; Fwavelength(1,24) = 1.188, p = 0.2866; Fwt vs mutant(5,24) = 1.282, p = 0.3041. Data are presented as mean ± S.D. from 3 replicates.
mutants, inositol, which does not contain phosphoryl groups, caused minimal changes (2–4%) in peak intrinsic fluorescence of h\(\beta N1\), h\(\gamma N2\), and h\(\gamma C\) in B; and mutant peptides in C. Changes in peak emissions with excitation at 250 (D) and 280 nm (E). Summary results for WT and mutant peptides shown as black and white histograms, respectively. Significant peak changes were determined by multiple t tests (*, \(p < 0.05\); **, \(p < 0.005\)). Peak changes of 5% or less were compared with mutant peak changes and analyzed by 2-way ANOVA (interaction(5,24) = 0.2827, \(p = 0.9180\); Fwavelength(1,24) = 0.0144, \(p = 0.9054\); Fwt vs mutant(5,24) = 2.4533, \(p = 0.0624\)), showing no significant difference. Data are presented as mean ± S.D. from 3 replicates.

**Discussion**

This is the first study that we are aware of, that directly quantifies the binding affinity of ENaC for phospholipids. Phospholipid binding to ENaC is thought to be critical to the proper function of the channel. Here, cellular and *in vitro* biophysical methods demonstrate that PIP\(_2\) regulates hENaC activity through direct interactions with at least three distinct intracellular domains of the channel. Each of these domains is rich in cationic residues. Results from the CRY2-CIBN optogenetics cellular assay show that depletion of PIP\(_2\) reduces intracellular Na\(^+\) levels in HEK cells transfected with ENaC, whereas Na\(^+\) levels remain constant in control cells lacking ENaC. Bioinformatics analysis was consistent with several cationic-rich regions within \(\beta\) and \(\gamma\)-ENaC cytoplasmic tails forming amphipathic helices and the clustering of positively charged residues to one side of these helices. Such clustering would form electropositive sites attractive to the negative phosphoryl...
groups of PIP$_2$. Such phosphoryl groups, as demonstrated by results shown in Fig. 2, are necessary for PIP$_2$ regulation of ENaC. Results from SSIF ligand-binding experiments were consistent with both PIP$_2$ and PIP$_3$ changing the secondary structure of intracellular domains of the channel proteins, specifically the extreme N terminus of $\beta$-ENaC ($\beta$N1) and a membrane adjacent region in the N terminus of $\gamma$-ENaC ($\gamma$N2); whereas, only PIP$_2$ significantly altered the membrane adjacent to the carboxyl terminus of $\gamma$-ENaC ($\gamma$C). Results from MST experiments demonstrate that PIP$_2$ binds to $\beta$N1 and $\gamma$N2 with dissociation constants in the lower micromolar range and $\gamma$C in the high micromolar range. In contrast, charge reversal of the cationic residues in these domains obliterated phospholipid binding. Together these results indicate that PIP$_2$, and possibly PIP$_3$, regulate ENaC activity by directly interacting with the $\beta$N1 and $\gamma$C regions of ENaC, but also circumvented the limitations of this earlier study, which was constrained by the necessity of functional expressing ENaC. Consequently, the current study was able to reveal, in addition, that $\gamma$N2 is also involved in interactions with PIP$_2$.

These results are consistent with the hypothesis that PIP$_2$ interacts with ENaC at three discrete cationic sites, without the need for an adapter molecule. Like ENaC, $K_+$ and TRP channels also contain multiple PIP$_2$-interaction sites that are distant from each other. Interestingly, these sites are joined together by a single PIP$_2$ molecule. For example, homology modeling of TRPV1 revealed that basic residues on the C terminus and the S4–S5 linker interact with a single PIP$_2$ headgroup (40). Cryo-EM analysis of TRPM8 shows a single PIP$_2$ molecule binding distant cationic residues within the pre-S1, S4–S5, TRP, and MHR4’ domains (25). Likewise, X-ray crystallography of GIRK/K$_{ir}$3.2 shows a single PIP$_2$ headgroup interacting with cationic residues at the C terminus-membrane interface and a more distant N-terminal lysine (22). If this binding mode generalizes to ENaC then the apparent presence of multiple
**PIP₂ interacts with multiple sites on ENaC**

PIP₂-binding sites within ENaC may actually reflect the possibility that distant domains come together to form a single binding site. In the absence of a resolved structure of the full channel with PIP₂, the formation of such a larger PIP₂-binding pocket remains speculative, and we can only conclude from the current results that there are at least three discrete PIP₂ interaction sites within ENaC.

Another key observation of the PIP₂-TRP and PIP₂-KCN structures is the high helical content at the PIP₂ interaction sites. As noted in Fig. 4, helical formation of the PIP₂-ENaC sites may form an electropositive region to enhance binding with the phosphoryl groups of PIP₂. A recent study reported that the purified γN terminus of ENaC adopted a helical conformation upon addition of a PIP₂ analog, consistent with PIP₂ inducing or stabilizing a helical conformation in γN-ENaC (31). The extreme N terminus of β-ENaC is also predicted to be helical. The βN1 site shares sequence homology with the N terminus of the Caenorhabditis elegans Mec-4 ion channel, which also is a member of the ENaC/Deg ion channel family (Fig. S4). This sequence similarity is notable because the solution NMR structure of Mec-4 displays several α helices in its N terminus in the absence of PIP₂ (PDB ID 2K2B) (48). This suggests that the related βN1 region of hENaC might also be helical prior to binding PIP₂, whereas the γN2 region may first be disordered then become helical after coming into contact with PIP₂. The impact of such structural changes is not yet obvious, but one thought is that any structural changes caused by PIP₂ to the cytoplasmic termini of ENaC could impact the open probability of the channel. This would be consistent with previous work where ENaC open probability was noted to be tied in a positive manner to membrane PIP₂ levels (14).

The major contribution of this work is the quantification of PIP₂-ENaC interactions. The low to moderate affinities of PIP₂-ENaC interactions revealed by MST experiments provide insight about the control of ENaC activity by membrane phospholipids. ENaC is constitutively active, requiring PIP₂ for this activity. This is known because ENaC activity quickly declines in response to a reduction in membrane PIP₂ levels, with all channels closed within 5 min (14, 28, 29). The moderate and low binding affinities of ENaC for PIP₂ documented in the current study possibly explain this critical sensitivity of ENaC for PIP₂. If \( K_d \) values had reflected more stringent binding, perhaps in the picomolar to nanomolar range, then PIP₂ would likely remain associated with the channel as unbound PIP₂ is depleted within the membrane, and the current rundown would not be observed, or would take much longer. The lower to moderate affinities reported here favor PIP₂ more quickly dissociating from ENaC in response to changes in membrane PIP₂ levels allowing such regulation to be dynamic.

Having only moderate affinity for PIP₂ might also contribute to tight spatiotemporal regulation of ENaC. Enrichment of PIP₂ in the plasma membrane perhaps is an effective method to maintain nascent channels quiescent until they reach their destination at the plasma membrane (19). Because other phosphoinositides, but not PIP₂, are present in intracellular vesicles, ENaC must have a high enough affinity to be selective for PIP₂ once it reaches the plasma membrane, yet not so high that it cannot properly respond to changes in PIP₂ levels. Thus, the low to moderate \( K_d \) values discovered by this study are consistent with patch clamp studies and explain why ENaC activity is highly responsive to membrane levels of PIP₂.

Tubby, the phospholipid reporter used in experiments reported in Fig. 1, can interact with other phospholipids, including PIP₃ (21, 41). Therefore the significant reduction we observed of membrane Tubby after BLI may also represent the depletion of other membrane phospholipids. PIP₃ is also present at the plasma membrane, and earlier studies had suggested a role for both PIP₂ and PIP₃ in the regulation of ENaC in cultured nonpolarized cells. Similar to PIP₂ effects on ENaC, addition of PIP₃ to a bath solution containing excised patches of ENaC enhances ENaC currents (14, 28). The results in this study are consistent with ENaC binding both phospholipids. The structure of PIP₃ is similar to that of PIP₂, with the former differing from the latter only by the presence of an additional phosphoryl group at carbon 3 of the inositol ring. This extra phosphoryl group gives PIP₃ an additional net +2 e charge at neutral pH, suggesting PIP₂ might bind more strongly to ENaC than PIP₂. However, despite these observations, strong evidence contradicts physiological interactions between PIP₃ and ENaC in polarized kidney cells, due to their distinct subcellular localization. For instance, storm imaging shows PIP₂ segregated into nanoclusters, or lipid rafts, distinct from PIP₃ nanoclusters (42). This might suggest that PIP₂ and PIP₃ would not simultaneously bind ENaC, and that ENaC would have to be in the same lipid raft as either phospholipid to support interactions. The same study reported, like others, that PIP₃ is present at much lower concentrations than PIP₂ in the plasma membrane, bolstering the case that PIP₂ would be more likely to interact with ENaC than PIP₃. Other studies performed in polarized Madin-Darby canine kidney cells and LLC-PK1 cells found that PIP₂ preferentially localizes to the apical membrane in polarized cells, whereas PIP₃ predominates in the basolateral membrane (43, 44). Because ENaC is also located in the apical membrane (10, 45), PIP₂, but not PIP₃, would most likely be the phospholipid that affects ENaC activity in polarized epithelial cells, such as the principal cells of the cortical collecting. Although we, and others, observe PIP₃-ENaC interactions in vitro, these subcellular localization patterns suggest that PIP₂ is the physiologically relevant binding partner, and careful interpretation of phospholipid interaction should be emphasized when studying these interactions in nonpolarized cells.

Because the dysfunction of ENaC contributes to hypertension and other diseases, such as cystic fibrosis, understanding the molecular mechanism of phospholipid regulation of ENaC is important. However, the difficulty of studying the full channel and lipid-based molecules in aqueous solutions has inhibited our ability to fully understand the dynamics of phospholipid-ion channel interactions. In this study, we used peptides and soluble phospholipid analogs to quantify PIP₂ interactions with ENaC. Quantifying such interactions as done here possibly explains how a low to moderate PIP₂ binding affinity for ENaC precisely controls channel activity at the apical plasma membrane of polarized epithelial cells.
**Experimental procedures**

**Plasmids, synthetic peptides, and reagents**

Plasmids containing mCherry-CRY2–5-phosphataseOCRL (mCh-CRY2–5-ptase) and CIBN-CAAX were gifts from Pietro De Camilli (Addgene plasmid numbers 66836 and 79574). The YFP-Tubby plasmid was a gift from Andrew Tinker (Queen Mary University of London, UK). Human ENaC constructs in pMT3 capable of expressing channel subunits in mammalian systems have been described previously (46, 47). For these studies, the cDNA sequence for α-hENaC was cloned in-frame into the pECFP-C1 (Clontech/Takara, USA) plasmid. The resulting eCFP-α-hENaC fusion protein was used to track expression.

Synthetic peptides of the cytoplasmic regions of β- and γ-hENaC subunits were designed according to a previous study of PIP2 regulation of ENaC with mutated or deleted amino acids (14) with the corresponding sequences shown in Table S1. Peptides were prepared at 95% purity (Peptide 2.0, Chantilly, VA). Lyophilized peptides were reconstituted in HBS buffer (150 mM NaCl and 20 mM HEPES, pH 7.4). The peptide concentration was quantified by amino acid analysis (Protein Chemistry Lab, Texas A&M, College Station, TX) or absorbance at 280 nm corrected for the appropriate extinction coefficient. Peptides were named for subunit isoform and relative location. Mutants of ENaC peptides containing alanine (Ala) substitutions in place of cationic residues had very low solubility and were unstable. Consequently, they could not be tested in the current work. In contrast, the “charge-reversed” mutants substituted with glutamic acid (E) used here showed good stability and were soluble in the same buffer as WT peptides. Thus, the charged reversed mutants were used in this study.

FuGENE HD (Promega) was used for transient transfection. HEK 293 cells were from ATCC (CRL-1573). The cell permeant sodium indicator, CoroNa Green AM (ThermoFisher Scientific), was used to monitor intracellular Na⁺ levels. Phosphoinositides, including fluorescein-PIP2-HG were purchased from Echelon Biosciences Inc. (Salt Lake City, UT) and dissolved in HBS or chelced MilliQ water. Phosphoinositide solutions were sonicated for 3–5 min prior to use. All experiments involving phospholipids were performed at concentrations below that of their critical micelle concentration.

**Quantification of changes in intracellular [Na⁺] with optogenetics and live cell imaging**

HEK 293 cells were seeded in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum on 35-mm uncoated glass bottom dishes. A day after seeding, cells were transfected with 0.25 μg/construct of pECFP-C1-α-hENaC, pMT3-β-hENaC, pMT3-γ-hENaC, CIBN-CAAX and mCh-CRY2–5-ptase per dish using FuGENE HD following standard protocols. Control cells were transfected with YFP-Tubby + CIBN-CAAX and mCh-CRY2–5-ptase or only ENaC constructs. Transfected cells expressing CIBN-CAAX and mCh-CRY2–5-ptase with and without ENaC were maintained in standard tissue culture conditions in the dark and in the presence of 10 μM amiloride for 24 h. Prior to imaging, cells were incubated in the dark at 37 °C for 30 min in serum-free Dulbecco’s modified Eagle’s medium containing 10 μM amiloride (Sigma), 5 μM CoroNa Green (Molecular Probes), and 0.04% Pluronic F-127 (Biotium). Cells were rinsed with PBS and then imaged using a ×60/1.45 TIRF oil objective or ×10/0.13 objective with ×1.5 amplification on an inverted Nikon Eclipse TE2000-U fluorescence microscope. To obviate premature dimerization of CRY2 with CIBN, spurious illumination in the blue wavelength was avoided with cells positive for expression of these heterologous proteins identified initially with a mercury light source for excitation through a mCherry filter set, ET560/40 (Chroma number 96365). OBIS FP pigtailed lasers (Coherent, Inc.) were used at 2% power for imaging and BLI, unless otherwise noted. TIRF imaging of YFP-Tubby was used to quantify the fluorescence at the membrane. YFP-Tubby was monitored at 514 nm and the CIBN/CRY2 dimerization was induced at 445 nm at 10% power for 10 s. For quantitation of ENaC-facilitated Na⁺ entry under TIRF, CRY2 dimerization with CIBN was driven with BLI pulses for 30 s at 445 nm with 300 ms illumination at a 1-Hz frequency. CoroNa Green and mCh-CRY2–5-ptase were imaged at 488 and 561 nm, respectively, before, immediately following, 5 and 10 min after BLI. The presence of ENaC expression as assayed with eCFP-α-hENaC fluorescence was confirmed during BLI pulsing. Images were captured with an Andor iXon Ultra camera and evaluated using Metamorph software (Molecular Devices). Fluorescence intensity data were analyzed using ImageJ with images presented as total cellular fluorescence corrected for background noise. Membrane mCh-CRY2–5-ptase was compared in cells with and without ENaC by subtracting the total fluorescence of the cell before BLI from that 10 min after BLI using ImageJ. The change in fluorescence was plotted in arbitrary units normalized to the starting fluorescence of each experiment. Analysis of each cellular experiment was performed on n = 10–12 individual cells.

**Steady-state intrinsic fluorescence**

Steady-state intrinsic fluorescence of synthetic peptides (at 250 nm in HBS) was quantified in the absence and presence of 10 μM phosphoinositide (diC4-PIP3 or diC4-PIP2) or inositol by exciting at 250 and 280 nm and scanning emissions between 260 and 450 nm through 6-nm band passes using a Photon Technologies International (PTI) Quantamaster spectrophotometer. Neither diC4-PIP3 nor diC4-PIP2 absorbs or emits at these wavelengths. Data were collected using Felix32 software version 1.10 (PTI/HORIBA Instruments) and then compiled in Excel using an R Studio text file organizer created by Aaron Horning (Stanford University, CA). Final data analysis was performed in GraphPad Prism 7. Steady-state intrinsic fluorescence data are presented as corrected intensity, protein fluorescence minus buffer fluorescence. Changes in peak intensities were determined by dividing the highest point of the larger peak by the highest point of the smaller peak. Statistical significance of SSIF data were determined by multiple t tests and nonadjusted p values are reported. Peptides with peak changes of 5% or less were compared with mutant peptides using 2-way ANOVA followed by Tukey’s test to confirm no significant changes in fluorescence.
PIP₂ interacts with multiple sites on ENaC

Microscale thermophoresis

For MST experiments, synthetic peptides were serially diluted with equal parts HBS up to 16 times. Peptide concentrations ranged from 0.1 to 1000 μM. Following addition of fluorescein-PIP₂-HG (final concentration 100 nM), samples were loaded into premium, low protein-binding glass capillaries (catalog number MO-K005 or MO-AK005, Nanotemper Technologies). Microscale thermophoresis was then measured using a Nanotemper Monolith 1.115 (Case Western Reserve University, Cleveland, OH) or a Nanotemper NT.Automated (UT Health San Antonio, TX). Data were recorded using the auto-detected functions of IR laser power to apply the heat gradient to each capillary and light emitting diode power to track fluorescence migration along the heat gradient. Data were fit to a 1:1 binding model in PALMIST using the “cold” function, which compares fluorescence data before application of the heat gradient, or the “Tjump” function, which is the difference between the cold fluorescence and fluorescence immediately after application of the heat gradient (38). Data plots were rendered in GUSSI (39).

Bioinformatics and statistical analysis

ClustalOmega (RRID: SCR_001591) was used to generate alignments of mouse and human ENaC subunits (Uniprot IDs: P37088, P51168, and P51170). Secondary structures for β- and γ-haENaC subunits were predicted using Jpred4 (RRID: SCR_016504) and HHPred (RRID: SCR_010276). Helical projection models were created using EMBOSS Pepwheel (RRID: SCR_018398).

The mean of 10–12 independent experiments of cell assays is shown as mean ± S.D., or S.E. for grouped experiments of n = 4–6 cells in each independent experiment, where indicated. SSIF data are shown as a mean ± S.D. of 3 independent experiments. MST data are shown as mean ± S.D. and dissociation constants represent the mean of 3 experiments, with error reported as parametric confidence interval derived from the error surface projection method, previously described in detail (38). Statistical significance for each experiment was determined using GraphPad Prism 7 using either a paired, two-tailed t test, multiple t test, or S.D. of 3 independent experiments of cell assays is shown as a mean ± S.D. and dissociation constants represent the mean of 3 experiments, with error reported as parametric confidence interval derived from the error surface projection method, previously described in detail (38). Statistical significance for each experiment was determined using GraphPad Prism 7 using either a paired, two-tailed t test, multiple t test, or S.D. of 3 independent experiments of cell assays is shown as a mean ± S.D. and dissociation constants represent the mean of 3 experiments, with error reported as parametric confidence interval derived from the error surface projection method, previously described in detail (38). Statistical significance for each experiment was determined using GraphPad Prism 7 using either a paired, two-tailed t test, multiple t test, or S.D. of 3 independent experiments of cell assays is shown as a mean ± S.D. and dissociation constants represent the mean of 3 experiments, with error reported as parametric confidence interval derived from the error surface projection method, previously described in detail (38). Statistical significance for each experiment was determined using GraphPad Prism 7 using either a paired, two-tailed t test, multiple t test, or S.D. of 3 independent experiments of cell assays is shown as a mean ± S.D. and dissociation constants represent the mean of 3 experiments, with error reported as parametric confidence interval derived from the error surface projection method, previously described in detail (38). Statistical significance for each experiment was determined using GraphPad Prism 7 using either a paired, two-tailed t test, multiple t test, or S.D. of 3 independent experiments of cell assays is shown as a mean ± S.D. and dissociation constants represent the mean of 3 experiments, with error reported as parametric confidence interval derived from the error surface projection method, previously described in detail (38). Statistical significance for each experiment was determined using GraphPad Prism 7 using either a paired, two-tailed t test, multiple t test, or S.D. of 3 independent experiments of cell assays is shown as a mean ± S.D. and dissociation constants represent the mean of 3 experiments, with error reported as parametric confidence interval derived from the error surface projection method, previously described in detail (38). Statistical significance for each experiment was determined using GraphPad Prism 7 using either a paired, two-tailed t test, multiple t test, or S.D. of 3 independent experiments of cell assays is shown as a mean ± S.D. and dissociation constants represent the mean of 3 experiments, with error reported as parametric confidence interval derived from the error surface projection method, previously described in detail (38). Statistical significance for each experiment was determined using GraphPad Prism 7 using either a paired, two-tailed t test, multiple t test, or S.D. of 3 independent experiments of cell assays is shown as a mean ± S.D. and dissociation constants represent the mean of 3 experiments, with error reported as parametric confidence interval derived from the error surface projection method, previously described in detail (38). Statistical significance for each experiment was determined using GraphPad Prism 7 using either a paired, two-tailed t test, multiple t test, or S.D. of 3 independent experiments of cell assays is shown as a mean ± S.D. and dissociation constants represent the mean of 3 experiments, with error reported as parametric confidence interval derived from the error surface projection method, previously described in detail (38). Statistical significance for each experiment was determined using GraphPad Prism 7 using either a paired, two-tailed t test, multiple t test, or S.D. of 3 independent experiments of cell assays is shown as a mean ± S.D. and dissociation constants represent the mean of 3 experiments, with error reported as parametric confidence interval derived from the error surface projection method, previous...
27. Estacion, M., Sinkins, W. G., and Schilling, W. P. (2001) Regulation of the epithelial sodium channel (ENaC). J. Biol. Chem. 292, 375–385 CrossRef PubMed
26. Han, B., He, K., Cai, C., Tang, Y., Yang, L., Heinemann, S. H., Hoshi, T., and Yokoyama, S. (2008) Identification of the epithelial sodium channel (ENaC) in cultured duct cells. J. Biol. Chem. 283, 36534–36542 CrossRef PubMed
25. Yin, Y., Le, S. C., Hsu, A. L., Borgnia, M. J., Yang, H., and Lee, S. Y. (2019) The classical inward rectifier K+ channel GIRK2: how and why? Annu. Rev. Biochem. 88, 411–443 CrossRef PubMed
24. Weixel, K. M., Edinger, R. S., Kester, L., Guerriero, C. J., Wang, H., Fang, L., Kleyman, T. R., Welling, P. A., Weisz, O. A., and Johnson, J. P. (2007) Phosphatidylinositol 4,5-bisphosphate influences parathyroid hormone 1 receptor activity. Am. J. Physiol. Renal Physiol. 295, F546–F563 CrossRef PubMed
23. Whorton, M. R., and MacKinnon, R. (2011) Crystal structure of the mammalian GIRK2 K+ channel. J. Gen. Physiol. 138, 1–19 CrossRef PubMed
22. Suh, B. C., and Hille, B. (2008) PIP2 is a necessary cofactor for ion channel activation and expression of the epithelial sodium channel (ENaC) in cultured duct cells. J. Biol. Chem. 283, 290, 530, 1–19 CrossRef PubMed
21. Alli, A. A., Bao, H. F., Liu, B. C., Yu, L., Aldrugh, S., Montgomery, D. S., Ma, H. P., and Stockand, J. D. (2008) Phosphatidylinositol 4,5-bisphosphate (PIP2) stimulates epithelial sodium channel activity in A6 cells. J. Biol. Chem. 277, 11965–11969 CrossRef PubMed
20. Pochynyuk, O., Bugaj, V., and Stockand, J. D. (2008) Physiologic regulation of the epithelial sodium channel by phosphatidylinositides. Curr. Opin. Nephrol. Hypertens. 17, 533–540 CrossRef PubMed
19. Yan, Y., Le, S. C., Hsu, A. L., Borgnia, M. J., Yang, H., and Lee, S. Y. (2019) Structural basis of “c”-subunits of the human epithelial sodium channel. Am. J. Physiol. 295, 1–19 CrossRef PubMed
18. Pochynyuk, O., Bugaj, V., and Stockand, J. D. (2008) Regulation of the epithelial Na+ channel (ENaC) by phosphatidylinositides. Am. J. Physiol. Renal Physiol. 290, F949–F957 CrossRef PubMed
17. Hilgemann, D. W., and Ball, R. (1996) Regulation of cardiac Na+ channel activity. Annu. Rev. Physiol. 58, 55–63 CrossRef PubMed
16. Weixel, K. M., Edinger, R. S., Kester, L., Guerriero, C. J., Wang, H., Fang, L., Kleyman, T. R., Welling, P. A., Weisz, O. A., and Johnson, J. P. (2007) Phosphatidylinositol 4,5-bisphosphate 5-kinase reduces cell surface expression of the epithelial sodium channel (ENaC) in cultured duct cells. J. Biol. Chem. 283, 36534–36542 CrossRef PubMed
15. Chu, A., and Stefani, E. (1991) Phosphatidylinositol 4,5-bisphosphate-induced Ca2+ release from skeletal muscle sarcoplasmic reticulum terminal cisternae: Ca2+ flux and single channel studies. J. Biol. Chem. 266, 7699–7705 PubMed
14. Pochynyuk, O., Bugaj, V., Sinkins, W. G., and Schilling, W. P. (2001) Regulation of the epithelial sodium channel (ENaC). J. Biol. Chem. 292, 375–385 CrossRef PubMed
13. Hansen, S. B. (2015) Lipid agonism: the PIP2 paradigm of ligand-gated ion channels. Biochim. Biophys. Acta 1851, 620–628 CrossRef PubMed
12. Weixel, K. M., Edinger, R. S., Kester, L., Guerriero, C. J., Wang, H., Fang, L., Kleyman, T. R., Welling, P. A., Weisz, O. A., and Johnson, J. P. (2007) Phosphatidylinositol 4,5-bisphosphate 5-kinase reduces cell surface expression of the epithelial sodium channel (ENaC) in cultured duct cells. J. Biol. Chem. 283, 36534–36542 CrossRef PubMed
11. Alli, A. A., Bao, H. F., Liu, B. C., Yu, L., Aldrugh, S., Montgomery, D. S., Ma, H. P., and Stockand, J. D. (2008) Phosphatidylinositol 4,5-bisphosphate (PIP2) stimulates epithelial sodium channel activity in A6 cells. J. Biol. Chem. 277, 11965–11969 CrossRef PubMed
10. Estacion, M., Sinkins, W. G., and Schilling, W. P. (2001) Regulation of Drosophila transient receptor potential-like (TrpL) channels by phospholipase C-dependent mechanisms. J. Physiol. 530, 1–19 CrossRef PubMed
9. Pochynyuk, O., Tong, Q., Staruschenko, A., Ma, H. P., and Stockand, J. D. (2006) Regulation of the epithelial Na+ channel (ENaC) by phosphatidylinositides. Am. J. Physiol. Renal Physiol. 290, F949–F957 CrossRef PubMed
8. Weixel, K. M., Edinger, R. S., Kester, L., Guerriero, C. J., Wang, H., Fang, L., Kleyman, T. R., Welling, P. A., Weisz, O. A., and Johnson, J. P. (2007) Phosphatidylinositol 4,5-bisphosphate 5-kinase reduces cell surface expression of the epithelial sodium channel (ENaC) in cultured duct cells. J. Biol. Chem. 283, 36534–36542 CrossRef PubMed
7. Estacion, M., Sinkins, W. G., and Schilling, W. P. (2001) Regulation of Drosophila transient receptor potential-like (TrpL) channels by phospholipase C-dependent mechanisms. J. Physiol. 530, 1–19 CrossRef PubMed
6. Pochynyuk, O., Bugaj, V., Sinkins, W. G., and Schilling, W. P. (2001) Regulation of the epithelial sodium channel (ENaC). J. Biol. Chem. 292, 375–385 CrossRef PubMed
5. Weixel, K. M., Edinger, R. S., Kester, L., Guerriero, C. J., Wang, H., Fang, L., Kleyman, T. R., Welling, P. A., Weisz, O. A., and Johnson, J. P. (2007) Phosphatidylinositol 4,5-bisphosphate 5-kinase reduces cell surface expression of the epithelial sodium channel (ENaC) in cultured duct cells. J. Biol. Chem. 283, 36534–36542 CrossRef PubMed
4. Weixel, K. M., Edinger, R. S., Kester, L., Guerriero, C. J., Wang, H., Fang, L., Kleyman, T. R., Welling, P. A., Weisz, O. A., and Johnson, J. P. (2007) Phosphatidylinositol 4,5-bisphosphate 5-kinase reduces cell surface expression of the epithelial sodium channel (ENaC) in cultured duct cells. J. Biol. Chem. 283, 36534–36542 CrossRef PubMed
3. Estacion, M., Sinkins, W. G., and Schilling, W. P. (2001) Regulation of Drosophila transient receptor potential-like (TrpL) channels by phospholipase C-dependent mechanisms. J. Physiol. 530, 1–19 CrossRef PubMed
2. Pochynyuk, O., Bugaj, V., and Stockand, J. D. (2008) Physiologic regulation of the epithelial sodium channel by phosphatidylinositides. Curr. Opin. Nephrol. Hypertens. 17, 533–540 CrossRef PubMed
1. Weixel, K. M., Edinger, R. S., Kester, L., Guerriero, C. J., Wang, H., Fang, L., Kleyman, T. R., Welling, P. A., Weisz, O. A., and Johnson, J. P. (2007) Phosphatidylinositol 4,5-bisphosphate 5-kinase reduces cell surface expression of the epithelial sodium channel (ENaC) in cultured duct cells. J. Biol. Chem. 283, 36534–36542 CrossRef PubMed

**PIP2 interacts with multiple sites on ENaC**