Regulation of the epithelial Na\textsuperscript{+} channel (ENaC) by phosphatidylinositides

Oleh Pochynyuk, Qiusheng Tong, Alexander Staruschenko, He-Ping Ma, and James D. Stockand

1Department of Physiology, University of Texas Health Science Center, San Antonio, Texas; and 2Division of Nephrology, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama

Pochynyuk, Oleh, Qiusheng Tong, Alexander Staruschenko, He-Ping Ma, and James D. Stockand. Regulation of the epithelial Na\textsuperscript{+} channel (ENaC) by phosphatidylinositides. Am J Physiol Renal Physiol 290: F949–F957, 2006; doi:10.1152/ajprenal.00386.2005.—The epithelial Na\textsuperscript{+} channel (ENaC) is an end-effector of diverse cellular signaling cascades, including those with phosphatidylinositide second messengers. Recent evidence also suggests that in some instances, phosphatidylinositides can directly interact with ENaC to increase channel activity by increasing channel open probability and/or membrane localization. We review here findings relevant to regulation of ENaC by phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) and phosphatidylinositol 3,4,5-triphosphate (PIP\textsubscript{3}). Similar to its actions on other ion channels, PIP\textsubscript{2} is permissive for ENaC openings having a direct effect on gating. The PIP\textsubscript{3} binding site in ENaC involved in this regulation is most likely localized to the NH\textsubscript{2} terminus of \(\beta\)-ENaC. PIP\textsubscript{3} also affects ENaC gating but, rather than being permissive, augments open probability. The PIP\textsubscript{2} binding site in ENaC involved in this regulation is localized to the proximal region of the COOH terminus of \(\gamma\)-ENaC just following the second transmembrane domain. In complementary pathways, PIP\textsubscript{3} also impacts ENaC membrane levels through both direct actions on the channel and via a signaling cascade involving phosphoinositide 3-OH kinase (PI3-K) and the aldosterone-induced gene product serum and glucocorticoid-inducible kinase. The putative PIP\textsubscript{3} binding site in ENaC involved in direct regulation of channel membrane levels has not yet been identified.

phosphatidylinositol 4,5-bisphosphate; phosphatidylinositol 3,4,5-triphosphate; receptor tyrosine kinase; insulin G protein-coupled receptor

ION CHANNELS PLAY CRITICAL roles in every aspect of physiology. Moreover, ion channels have long been recognized to be important end-effectors of diverse cellular signaling cascades, including those having phosphatidylinositide second messengers. Direct regulation of ion channel activity by phosphatidylinositide-signaling molecules, in addition, is now becoming widely appreciated (reviewed in Ref. 33). This mechanism for ion channel modulation is recognized to be physiologically important, for its disruption, in some instances, leads to disease (e.g., Bartter’s and Andersen’s syndromes) (16, 43, 56, 65). Diverse types of ion channels are directly modulated by phosphatidylinositides (3, 17, 58, 74, 96, 97). Phosphatidylinositides, such as phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) and phosphatidylinositol 3,4,5-triphosphate (PIP\textsubscript{3}), directly interact with these channels to modulate gating (58, 93, 97). Several recent studies identify the epithelial Na\textsuperscript{+} channel (ENaC) as being a channel sensitive to direct phosphatidylinositide regulation.

ENaC is a heteromeric channel composed of three distinct but similar subunits: \(\alpha\), \(\beta\), and \(\gamma\) (8, 9). All ENaC subunits have NH\textsubscript{2}- and COOH-terminal cytosolic domains separated by two transmembrane domains and a large extracellular region. ENaC serves an essential physiological function, for its activity is limiting for Na\textsuperscript{+} absorption across many epithelia, including that in the distal renal nephron (reviewed in Refs. 27, 37, and 69). Thus ENaC is well positioned to influence systemic Na\textsuperscript{+} balance and blood pressure. Indeed, gain and loss of ENaC function lead to inheritable forms of hypertension and hypotension, respectively, associated with inappropriate salt conservation and wasting at the kidney (35, 41). In addition, ENaC plays a critical role in hydration and fluid reabsorption across many mucosal membranes. Deletion and overexpression of ENaC correspondingly lead to excessively wet and dry air spaces, with associated disease (35, 46).

Here, we summarize recent findings and current thinking regarding phosphatidylinositide regulation of this important ion channel. Both the regulation of ENaC by signaling cascades utilizing PIP\textsubscript{3} as a second messenger and the direct effects of PIP\textsubscript{2} and PIP\textsubscript{3} on ENaC are discussed. Where possible, the physiological implications of recent findings are addressed.

**REGULATION OF ENaC BY PIP\textsubscript{2}**

**PIP\textsubscript{2} Increases ENaC Open Probability**

Most ion channels sensitive to PIP\textsubscript{2}, including the first channel identified to be sensitive to this signaling molecule, K\textsubscript{ATP} (Kir6.2/SUR2) (32), have a continuing decrease in activity when excised from the cell membrane in an inside-out patch configuration (reviewed in Refs. 33 and 85). This hallmark is referred to as channel “run-down.” In excised, inside-out patches, ENaC shows run-down typical to PIP\textsubscript{2}-sensitive channels (44, 95).

Ma and colleagues (44) provided the first clear evidence that ENaC is directly regulated by phosphatidylinositides. This
group demonstrated that scavenging PIP$_2$ with a specific antibody and poly-L-lysine accelerated ENaC run-down in excised inside-out patches made from the amphibian distal tubule A6 cell line. Similarly, increased PIP$_2$ hydrolysis in response to activation of endogenous PLC via signaling from purinergic receptors and addition of exogenous PLC accelerated ENaC run-down. Run-down was markedly slowed by addition of PIP$_2$ to the cytosolic bath solution bathing the intracellular face of the channel. The primary effect of PIP$_2$ on ENaC was not related to changes in the membrane levels of this channel within 30 min. Thus the authors concluded that acute regulation of ENaC by PIP$_2$ is independent of channel trafficking. This work suggests that PIP$_2$ augments ENaC open probability through direct interactions with intracellular/membrane portions of the channel or through interactions with a phosphatidylinositide-sensitive, intracellular regulator of the channel.

Several investigators have subsequently confirmed possible direct effects of PIP$_2$ on ENaC in excised, inside-out patches (39, 88, 95). Figure 1 shows a representative finding demonstrating PIP$_2$ activation of ENaC in an excised, inside-out patch (Tong Q and Stockand JD, unpublished observations). An interesting extension to the original finding by Ma and colleagues (44) was made by Yue and colleagues (95). This group demonstrated that PIP$_2$ had only modest effects on ENaC in excised, inside-out patches from A6 cells compared with the effects of this phosphatidylinositide when added in combination with GTP. The primary action of PIP$_2$+GTP was to reverse run-down by increasing channel open probability to levels approaching starting values.

Addition of activated G$_{G_{i-3}}$ and G protein $\beta\gamma$-subunits, in the study by Yue and coauthors, mimicked and reversed the actions of GTP, respectively, implicating involvement of a trimeric G protein in a PIP$_2$ response. We have reported a similar finding where dialysis of intracellular GTP with GDP$\beta$S quickly decreases activity of mouse and human ENaC heterologously expressed in Chinese hamster ovary (CHO) cells in the absence and presence of activated phosphoinositide 3-OH kinase (PI3-K) (57). PI3-K activates ENaC via PIP$_3$ signaling (see below). We attribute the action of GDP$\beta$S to inhibition of either a Ras or Rho small G protein (57, 77–79). Thus G proteins in a yet to be fully appreciated manner play a critical role in how ENaC responds to phosphatidylinositides.

There is precedence for interactions between G proteins and phosphatidylinositide regulation of ion channels. For G protein-activated, inwardly rectifying K$^+$ channel (GIRK), it is probable that PIP$_2$ and G protein $\beta\gamma$-subunits bind to the
COOH terminus of the channel in close proximity (62, 73). The closeness of these binding sites provides a platform for PIP2 and G protein βγ-subunits to interact with respect to regulating the channel. It currently is not clear whether G proteins directly interact with ENaC; however, PIP2 does (see below).

Two recent studies investigated regulation of ENaC by PIP2 using experimental approaches not complicated by channel run-down. In the first, Kunzelmann and colleagues (39) showed that inhibition in response to purinergic receptor signaling of Na+ absorption mediated by ENaC across tracheal epithelia and M1 collecting duct monolayers was suppressed by scavenging PIP2. Recovery of inhibition in response to purinergic receptor signaling, moreover, was abolished by blocking PI4-K and diacylglycerol kinase. Both PI4-K and diacylglycerol kinase are involved in PIP2 synthesis (24).

We recently demonstrated that ENaC activity in whole cell and excised, outside-out patch configurations decreases on depletion of membrane PIP2 in response to activation of receptor tyrosine kinase signaling and signaling via a classic Gq/11-coupled seven-transmembrane-spanning receptor (88). In contrast to the excised, inside-out patch configuration, ENaC does not run down in either of these latter patch configurations (6, 79, 87, 88). PIP2 levels were likely depleted by receptor tyrosine kinase- and Gq/11-coupled signaling via PLC-γ and -β, respectively. In excised, outside-out patches, depletion of PIP2 decreased ENaC activity by decreasing open probability. As expected, buffering PIP2 to prevent dynamic changes in the levels of this phosphatidylinositide counteracted decreases in ENaC activity in response to receptor tyrosine kinase signaling. Thus the response of ENaC to PIP2 depletion mediated by receptor tyrosine kinase-PLC-γ and Gq/11-PLC-β parallels regulation of tryptophan (TRP), P/Q-, and N-type Ca2+ and GIRK channels by receptor tyrosine kinases (13, 38, 58, 92), and KCNQ and N-type Ca2+ channels by Gq/11-coupled receptors (26, 40, 85, 92).

We propose that the effects of PIP2 on ENaC open probability are permissive rather than regulatory. Our rationale is that ENaC quickly runs down in settings such as excised inside-out patches and activation of PLC, where membrane PIP2 levels rapidly decline in response to uninhibited hydrolysis and/or washout (39, 44, 47, 88, 95). In contrast, in settings, such as whole cell and excised, outside-out patch configurations, where PIP2 levels are expected to be relatively constant, ENaC does not run down (6, 79, 87, 88). Moreover, ENaC activity (and open probability) never exceeds the levels of ENaC heterologously expressed in CHO cells rather than ENaC in the Rho family increase ENaC activity when they are overexpressed together in CHO cells (77). Rho increases channel activity by increasing the membrane levels of ENaC. PI(4)5-K is a downstream effector of Rho and Rho-kinase, the close relation being with respect to actions on ENaC are saturated at rest. Supporting such a mechanism is the fact that PIP2 is the most abundant phosphatidylinositide in the membrane, exceeding the levels of PIP2 by up to 100-fold (4, 24, 52).

**PIP2 Directly Interacts with ENaC**

Two studies have begun to delineate the PIP2 binding site in ENaC that impacts channel open probability (39, 95). Both suggest that PIP2 binds to a region in β-ENaC found in the cytosolic NH2 terminus of this subunit. As shown in Fig. 2A, this region of the channel contains several well-conserved, positively charged arginines and lysines. In this regard, then, the putative PIP2 binding site in ENaC is similar to that proposed for Kir and TRP channels, which all have an amino acid track containing several well-conserved, positively charged residues that potentially facilitate electrostatic interactions with the polar head groups of the phosphatidylinositides in the inner leaflet of the plasma membrane (17, 58, 66, 73, 97). Mutation of this domain in β-ENaC reduces ENaC activity without affecting surface expression (39).

**PIP2 Also Affects Membrane Levels of ENaC**

Vesicle movement and membrane targeting in eukaryotic cells are intimately tied to PIP2 (14, 19, 67). Small G proteins in the Rho family increase ENaC activity when they are overexpressed together in CHO cells (77). Rho increases channel activity by increasing the membrane levels of ENaC. PI(4)5-K is a downstream effector of Rho and Rho-kinase, the Rho effector (54, 91, 94). Overexpression of activated Rho-kinase and PI(4)5-K, similar to Rho, increases ENaC activity. Inhibition of Rho-kinase and PI4-K disrupts the effect of Rho on ENaC. Thus in CHO cells at least, Rho increases ENaC activity by increasing the membrane levels of this channel via chronic overexpression of PI(4)5-K, which is a phospholipid kinase that promotes PIP2 synthesis, increased membrane levels of ENaC heterologously expressed in CHO cells rather than the channel’s open probability (77) (see below). Thus we propose that active ENaC constitutively interacts with membrane PIP2, with this interaction being permissive for normal channel gating. Loss of PIP2 then would decrease open probability, but increases in PIP2 might not be expected to affect open probability if membrane levels of this phosphatidylinositide with respect to actions on ENaC are saturated at rest. Supporting such a mechanism is the fact that PIP2 is the most abundant phosphatidylinositide in the membrane, exceeding the levels of PIP3 by up to 100-fold (4, 24, 52).

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**Fig. 2.** PIP2 and phosphatidylinositol 3,4,5-triphosphate (PIP3) binding sites in ENaC. Shown are sequence alignments of the putative PIP2 (A) and PIP3 (B) binding sites in β- and γ-ENaC, respectively. Amino acid number is for mouse (m)ENaC. ch, Chimpanzee; r, rat; ra, rabbit; c, dog; x, *Xenopus laevis.*
a signaling cascade involving Rho-kinase, PI(4,5)-K, and its product, PIP2. We speculate that a sustained increase in PIP2 levels increases membrane ENaC through a mechanism independent of direct phosphatidylinositol binding to the channel. This mechanism likely is a more generalized trafficking response to chronic changes in PIP2. The physiological importance of PIP2 regulation of ENaC membrane levels via a trafficking response in epithelial cells has not yet been determined.

In summary, decreases in ENaC open probability parallel decreases in PIP2 levels. In contrast, increases in ENaC membrane levels parallel increases in PIP2 levels. Thus, as shown in the flow chart in Fig. 3, PIP2 may have multiple actions on ENaC to control channel activity.

**REGULATION OF ENaC BY PIP3**

**PIP3 is a Second Messenger of an Aldosterone-Sensitive Cellular Signaling Cascade that Impacts ENaC Activity**

ENaC is a critical end-effector of the renin-angiotensin-aldosterone system involved in negative-feedback control of blood pressure. Aldosterone increases ENaC activity in epithelia (reviewed in Refs. 27, 41, 82, and 89). Aldosterone increases both the number of ENaC in the plasma membrane and the open probability of the channel to increase the number of active channels in the membrane. Paunesku and colleagues (55) demonstrated that aldosterone activates PI3-K to increase PIP3 levels in renal epithelia and that active PI3-K is required for aldosterone actions on Na+ transport mediated by ENaC. Several laboratories have subsequently confirmed the observations that aldosterone activates PI3-K in renal epithelia and that active PI3-K is necessary for increases in Na+ transport in response to aldosterone (22, 86, 90). Two other natriuretic factors, insulin and antidiuretic hormone (ADH), also activate PI3-K in epithelia and increase Na+ absorption mediated by ENaC in a PI3-K-sensitive manner (20, 47, 48, 59). Importantly, insulin, while targeting basolateral receptors, quickly increases apical membrane PIP3 levels to affect activity of ENaC in this latter membrane (5). Active PI3-K also plays a role in setting basal ENaC activity in the absence of hormone stimulation (4, 55, 59). These findings suggest that active PI3-K and its phospholipid products are either permissive for ENaC activity or involved in regulation of both basal and hormone-sensitive ENaC activity. As expected, overexpression of active PI3-K with ENaC in CHO cells increased channel activity (79, 86, 87). Similarly, addition of membrane-permeant PIP3 increased Na+ absorption across renal epithelia, and overexpression of a PIP3-phosphatase, phosphatase and TENsin homolog deleted on chromosome 10, attenuated the natriuretic effects of insulin and exogenous PIP3 (47). Activation of PI3-K, moreover, with H2O2 provoked a rise in Na+ transport (48). Thus PI3-K activity is intimately tied to ENaC activity.

The mechanism by which aldosterone activates PI3-K in epithelia remains uncertain. We (31, 83) and others (49, 75, 76) demonstrated that in renal epithelia, aldosterone via control of gene expression increases the levels and activity of the small G protein K-Ras. PI3-K is a well-documented first effector of Ras (2, 60). In a recent publication (86), we argued that activation of PI3-K was a consequence of increased K-Ras expression in response to aldosterone.

Active PI3-K has been shown to modulate ENaC activity through, at least, three mechanisms (see Fig. 3). Two involve increases in the membrane levels of the channel, and the other involves increases in the open probability of the channel. As described further below, the product of PI3-K, PIP3, affects ENaC membrane levels by both a direct mechanism involving physical association of the phosphatidylinositol with the channel (30) and an indirect mechanism involving a signaling cascade that impinges on channel retrieval (15, 70). Regulation of ENaC open probability by PI3-K is a direct consequence of the physiological association of PIP3 with the channel (57, 87).

**PIP3 Signaling Impacts ENaC Membrane Levels by Suppressing Channel Retrieval**

The most studied and thus best-documented mechanism by which PI3-K signaling impacts ENaC activity involves suppression of channel retrieval by downstream effectors of this kinase. This mechanism is the focus of many excellent reviews (36, 63, 68, 69) and thus is covered only briefly here.

Membrane levels of ENaC are set, in part, by mediated channel retrieval. Ubiquitin ligases in the Nedd4 family interact with the conserved PY motifs in the cytosolic COOH-terminal domains of ENaC subunits (1, 29, 72, 80, 81). This interaction facilitates ubiquitylation of the channel, targeting it for internalization and subsequent degradation. Sgk is a serine/threonine kinase that is regulated at the level of transcription by corticosteroids, including aldosterone (10, 11, 51). This kinase also contains a PY motif and physically interacts with Nedd4 ubiquitin ligases (15, 70, 98). Association of Sgk with Nedd4 ubiquitin ligases leads to phosphorylation of the latter. This compromises the ability of Nedd4 ubiquitin ligases to bind and regulate ENaC. Thus activation of Sgk promotes retention of ENaC in the membrane. Sgk is a downstream effector of PI3-K sensitive to both PIP3 levels and phosphorylation by another PI3-K effector, PKD1 (22, 86, 90). Activation of PI3-K promotes activation of Sgk and subsequent protection of membrane ENaC levels. Aldosterone likely increases both Sgk levels and activity, with the former being a transcriptional event and the latter a signaling event in response to prior activation of PI3-K. The physiological importance of
this PI3-K-sensitive mechanism controlling ENaC membrane levels is clear. Disruption of the PY motifs in ENaC subunits leads to gain-of-function mutations associated with increased channel activity, improper Na$^+$ handling by the kidney and hypertension in humans (1, 25, 72, 80, 81). Mutation of the genes encoding Nedd4 and Sgk, moreover, has recently been linked to blood pressure imbalances and inappropriate renal electrolyte handling in humans (7, 23).

Blazer-Yost and colleagues (5) recently proposed an interesting mechanism for insulin modulation of Na$^+$ reabsorption in renal epithelia that has many parallels to regulation of ENaC by aldosterone. Both are dependent on increases in PI3-K activity, ultimately increasing ENaC activity. Blazer-Yost and colleagues proposed that activation of basolateral PI3-K in response to insulin signaling stimulates PIP3 production in this membrane, with the phosphatidylinositide rapidly diffusing within the inner membrane leaflet across tight junctions to the apical membrane. Increases in luminal PIP3 then increase ENaC activity by increasing the number of functional channels in the membrane. The importance of this signaling pathway is that it contains a possible mechanism for rapidly coupling basolateral signaling events to changes in activity of luminal ion channels. The rapidity of this putative signaling pathway arises from PIP3 diffusing within a planar lipid surface rather than a three-dimensional cystolic space. It is unclear whether the increase in ENaC activity observed in this study resulted from direct effects of the phosphatidylinositide on ENaC membrane levels and/or gating or indirect effects on ENaC insertion and/or retrieval.

**PIP3 Directly Interacts with ENaC to Increase Membrane Levels of the Channel**

In addition to influencing membrane levels of ENaC through a cell-signaling cascade, impinging on channel retrieval, PI3-K and its product PIP3 influence membrane levels of this channel through a more direct but less well-understood mechanism. Helms and colleagues (30) demonstrated that PIP3 binds ENaC at a yet to be defined domain, or a protein closely associated with the channel, to promote movement of the channel to the membrane. Currently, this mechanism appears to be distinct from that involving Sgk and Nedd4 proteins. However, further investigation is required to better substantiate this. That these experiments were performed in a renal epithelial cell line increases their importance. A domain near the NH$_2$ terminus of $\gamma$-ENaC that contains several conserved positively charged residues was found to be critical for PIP3 to increase ENaC activity and targeting of the channel to the membrane; however, this region appears not to be involved in PIP3 binding to the channel. Interestingly, this domain is similar in locale and charge to the PIP2 binding domain in $\beta$-ENaC. The importance of this similarity, as well as the exact function of this domain in $\gamma$-ENaC, however, remains unclear.

**PIP3 Directly Interacts with ENaC to Increase Open Probability**

As mentioned above, PI3-K is a first effector of Ras small G proteins (2, 60). In some epithelia, K-Ras is an aldosterone-induced protein necessary for steroid actions on ENaC open probability (49, 75, 83). Overexpression of K-Ras with ENaC in CHO cells increases channel activity independently of effects on ENaC membrane levels (78). K-Ras actions on ENaC are blocked by the PI3-K inhibitor wortmannin and mimicked by overexpression of active PI3-K (78, 79, 87). Overexpression of an effector-specific mutant of Ras capable of only activating PI3-K also increases ENaC activity in a wortmannin-sensitive manner (79). These results show that the aldosterone-induced protein, K-Ras, increases ENaC open probability by stimulating PI3-K.

The first indication that the phosphatidylinositide products of PI3-K, PIP3 and/or phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P$_3$], might directly interact with ENaC to modulate channel open probability came from experiments reporting parallel but fast changes in the active state of PI3-K, membrane PIP3/PI(3,4)P$_2$ levels, and ENaC activity in CHO cells (87). ENaC activity increased simultaneously with increases in membrane PIP3/PI(3,4)P$_2$ levels in response to both relief of PI3-K from inhibition and stimulation of PI3-K with IGF-I. Moreover, addition of exogenous PIP3 to the cytosolic face of inside-out patches containing ENaC that had run down increased channel activity (87). Addition of exogenous PIP3 and PI(3,4)P$_2$ to outside-out patches containing ENaC that had not run down increased channel activity above control levels (79, 87). These increases in activity resulted from definitive changes in open probability. Consistent findings have been reported for ENaC expressed in oocytes, where exogenous PIP3 increases channel activity without affecting membrane levels (44). Thus PIP3 directly regulates ENaC to increase activity and open probability above starting levels. Consistent with a direct interaction with the channel, a PIP3/PI(3,4)P$_2$ binding site in ENaC has now been described (57; see below). Disruption of this binding site counters PI3-K actions on ENaC activity, as well as disrupts increases in ENaC open probability in response to addition of exogenous PIP3. These findings thus demonstrate that similar to its actions on Kir channels (21, 45, 61, 62), PIP3 physically associates with ENaC to affect open probability.

We believe that in contrast to PIP2, the direct interaction of PIP3 with ENaC is regulatory rather than permissive. Our rationale is that PI3-K is a central component of several signaling cascades that increase ENaC activity above basal levels, and exogenous PIP3 increases channel activity above control levels. In addition, one effect of aldosterone and K-Ras is to increase ENaC open probability, an effect possibly mediated by PI3-K and direct interaction of PIP3 with the channel (as discussed above). Moreover, addition of exogenous PIP3 has little further effect on ENaC activity in renal epithelial cells pretreated with aldosterone but does significantly increase channel activity in cells not pretreated with steroid (30). Reports that membrane PIP3 levels are low compared with those of PIP2 and that aldosterone increases PIP3 levels are also consistent with a regulatory role for PIP3. However, it is also clear that PI3-K plays a role in maintaining basal ENaC activity for inhibition of this kinase rapidly decreases ENaC activity in epithelial cells not treated with steroid (55, 59). Possible involvement of a direct interaction between PIP3 and ENaC in setting basal channel activity remains to be fully explored.
**PIP3 Binding Sites in ENaC**

The PIP3/PI(3,4)P2 binding site in ENaC involved in regulation of channel open probability includes a region in the COOH-terminal cytosolic domain of γ-ENaC (57). This region is just distal to the second transmembrane domain in this subunit. Similar to the putative PIP2 binding site in the NH2 terminus of β-ENaC, this region of ENaC, as show in Fig. 2B, contains several well-conserved, positively charged arginine and lysine residues. Deletion of this region disrupts physical association of PIP3 with ENaC, as well as activation of the channel by PIP3 (57). Point mutation of the conserved positively charged residues in this region also disrupts regulation of the channel by PI3-K. This PIP3 binding site in ENaC then is similar to the phosphatidylinositol binding site in other channels in the respect that they all contain several positively charged conserved residues (17, 58, 66, 73, 97). Moreover, the location of this PIP3 binding site in ENaC just distal to the second transmembrane domain is ideally suited to modulate open probability. This locale just after a pore-lining transmembrane domain is also similar to the position of the phosphatidylinositol binding site in Kir and TRP channels.

An interesting additional observation made in this study, as reillustrated in Fig. 4, was that alanine substitution of the negatively charged aspartic acid and bulky tryptophans within the PIP3 binding site in γ-ENaC increased both basal and PI3-K-responsive ENaC activity. This suggests that these bulky and negatively charged residues may impact binding affinity or specificity. This possibility is similar to the role played by some noncharged and negatively charged residues in the binding sites of other phosphatidylinosine-sensitive channels. It has not yet been tested whether PIP2 also interacts with ENaC at this site. However, deletion of this site does decrease basal open probability, possibly indicating permissive actions of PIP2 or PIP3 binding.

**IMPLICATIONS OF SPATIAL AND TEMPORAL REGULATION OF PHOSPHATIDYLINOSITIDE TURNOVER FOR CONTROL OF ENaC**

It is becoming clear that similar to other second messengers, such as cAMP and Ca2+, which regulate myriad diverse cellular activities, PIP2 and PIP3 synthesis and metabolism must be both spatially and temporally regulated (reviewed in Refs. 18 and 53). Such regulation allows for localized subcellular changes in phosphatidylinositol levels, leading to dynamic and versatile phosphatidylinositol signaling. This is particularly important in polarized cells, which have the potential for distinct phospholipid compositions in specialized membranes and membrane areas. For instance, enzymes that synthesize and metabolize PIP3 are polarized in macrophages during chemotaxis, leading to a membrane PIP3 gradient (34). During fibroblast migration, moreover, membrane PIP3 levels show localized changes in response to PDGF (64). Similarly,

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**Fig. 4.** Conserved positively and negatively charged residues, as well as bulky aromatic amino acids in a putative PIP3 binding site in γ-ENaC modulate phosphoinositide 3-OH kinase (PI3-K) regulation of ENaC. A: summary graph showing ENaC activity in CHO cells in the absence (black bars) and presence of coexpression of constitutively active PI3-K (gray bars). Cells expressed either wild-type αβγ-ENaC or wild-type αβγ-ENaC plus γ-ENaC containing the indicated (bold) alanine-substituted amino acids. *Vs. activity in the absence of PI3-K. **Mutant ENaC + PI3-K significantly less than wild-type ENaC + PI3-K. B: summary graph showing ENaC activity in the absence (black bars) and presence of coexpression of constitutively active PI3-K (gray bars). Cells expressed wild-type αβγ-ENaC or wild-type αβγ-ENaC plus γ-ENaC containing the indicated alanine-substituted aspartic acid and tryptophan residues. *Activity of mutant ENaC in the absence and presence of PI3-K vs. wild-type ENaC in the absence and presence of PI3-K, respectively. All groups had significantly more current in the presence of PI3-K vs. its absence. Reprinted from Ref. 57 with permission.
the subcellular localization of PI3-K and PIP3 play critical roles in axon specification during neurite elongation and polarization (50). In response to insulin signaling, PIP3 synthesized in the basolateral membrane quickly diffuses to the apical membrane in renal epithelia containing ENaC (5). The translation and development of a PIP3 gradient correlate with increases in channel activity. Similar to PIP3, PIP2 accumulates in plasmalemmal microdomains (28). These membrane patches exhibit locally regulated PIP2 turnover and reduced diffusion, as well as accumulation of specialized proteins involved in membrane trafficking, signaling, and polarization. Localized rather than global changes in membrane phosphatidylinositol levels are presently thought to underlie many forms of ion channel regulation, as exemplified by Gq-coupled receptor regulation of GIRK (12).

Spatial organization of phosphatidylinositides within cellular membranes arises from the localized expression and/or activation of the kinases and phosphatases involved in synthesis and metabolism, as well as the localization of specific phospholipid precursors (18, 53). Temporal regulation arises from the activity of upstream regulators and the structure of these kinases and phosphatases. Recent investigation of phosphatidylinositol regulation of ENaC is consistent with temporal and spatial regulation of PIP2 and PIP3 playing important roles in modulation of this channel.

Although few studies, as yet, have specifically tested the effects of localized phosphatidylinositol turnover on ENaC activity, many predictions can be made from available results. For instance, regulation of ENaC open probability by PIP2 and PIP3 is predicted to be a localized rather than global response. Our rationale is that ENaC is found only in the apical membrane, and PIP2 and PIP3 likely directly interact with the channel to affect gating. Thus it ultimately is the phosphatidylinositol levels in the luminal membrane and more specifically near the channel that are important for phosphatidylinositol modulation of gating. The finding that PIP3 synthesized in response to insulin translocates to the luminal membrane is consistent with a localized response (5). Our finding that the aldosterone-induced protein K-Ras localizes PI3-K near ENaC is also consistent with tight special coupling between the channel and localized changes in membrane phosphatidylinositol levels (79). Findings in excised patches from both native epithelial and reconstituted systems documenting decreases in ENaC activity and open probability on activation of receptors coupled to PIP2 hydrolysis mandate, at least in these experimental settings, a localized response (39, 44, 88). Thus the cellular machinery apparently necessary for regulation of ENaC by local phosphatidylinositol turnover is present in many of the cases studied. Moreover, the tight temporal coupling of membrane phosphatidylinositol levels with changes in ENaC gating also suggests a localized rather than a global response (39, 44, 88).

It is more difficult to make predictions about whether local or global changes in PIP2 and PIP3 affect ENaC membrane levels. However, it is becoming clear that the effects of these phosphatidylinositolides on cellular trafficking are tightly controlled both spatially and temporally (18, 53). Thus it is also likely that PIP2 and PIP3 regulation of ENaC membrane levels is a response with specific subcellular check points, leading to a targeted and localized effect. However, compared with regulation of gating, regulation of ENaC membrane levels by phosphatidylinositolides appears to develop more slowly. We excitedly await further determination of the physiological relevance of spatial and temporal control of cellular phosphatidylinositol levels with respect to control of ENaC activity.

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