Regulation of the epithelial Na\(^+\) channel (ENaC) by phosphatidylinositides

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Am J Physiol Renal Physiol 290: F949–F957, 2006; doi:10.1152/ajprenal.00386.2005.—The epithelial Na\(^+\) 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\(_2\)) and phosphatidylinositol 3,4,5-triphosphate (PIP\(_3\)). Similar to its actions on other ion channels, PIP\(_2\) is permissive for ENaC openings having a direct effect on gating. The PIP\(_3\) binding site in ENaC involved in this regulation is most likely localized to the NH\(_2\)-terminus of \(\beta\)-ENaC. PIP\(_3\) also affects ENaC gating but, rather than being permissive, augments open probability. The PIP\(_2\) 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\(_3\) 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\(_2\) 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, 42, 58, 74, 96, 97). Phosphatidylinositides, such as phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) and phosphatidylinositol 3,4,5-triphosphate (PIP\(_3\)), directly interact with these channels to modulate gating (58, 93, 97). Several recent studies identify the epithelial Na\(^+\) channel (ENaC) as being a channel sensitive to direct phosphatidylinositol regulation.

ENaC is a heteromeric channel composed of three distinct but similar subunits: \(\alpha\), \(\beta\), and \(\gamma\) (8, 9). All ENaC subunits have NH\(_2\)- 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\(^+\) 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\(^+\) 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 phosphatidylinositol regulation of this important ion channel. Both the regulation of ENaC by signaling cascades utilizing PIP\(_3\) as a second messenger and the direct effects of PIP\(_2\) and PIP\(_3\) on ENaC are discussed. Where possible, the physiological implications of recent findings are addressed.

REGULATION OF ENaC BY PIP\(_2\)

**PIP\(_2\) Increases ENaC Open Probability**

Most ion channels sensitive to PIP\(_2\), including the first channel identified to be sensitive to this signaling molecule, K\(_{ATP}\) (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\(_2\)-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$_{i}$-3 and G protein $\beta y$-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 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 $\beta y$-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 Gq11-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 Gq11-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 phosphatidylinositol counted 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 Gq11-PLC-β parallels regulation of tryptophan (TRP), P/Q-, and N-type Ca\(^{2+}\) and GIRK channels by receptor tyrosine kinases (13, 38, 58, 92), and KCNQ and N-type Ca\(^{2+}\) channels by Gq11-coupled receptors (26, 40, 85, 92).

We propose that the effects of PIP2 on ENaC open probability are permissive rather then 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 control levels of this phosphatidylinositol 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 Gq11-PLC-β parallels regulation of tryptophan (TRP), P/Q-, and N-type Ca\(^{2+}\) and GIRK channels by receptor tyrosine kinases (13, 38, 58, 92), and KCNQ and N-type Ca\(^{2+}\) channels by Gq11-coupled receptors (26, 40, 85, 92).

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A finding that elevations of membrane PIP2 levels increase ENaC activity above control values would distinguish whether this phosphatidylinositol is permissive or regulatory. To date, all experiments reporting PIP2 actions on ENaC open probability have demonstrated a close link between decreases in open probability and declining PIP2 levels. The converse of increasing (above starting levels) open probability linked to elevations in PIP2 levels has not been established. Indeed, increasing cellular PIP2 levels by direct addition of a permeant form of this phosphatidylinositol failed to increase Na\(^+\) absorption mediated by ENaC in renal epithelia (47). Moreover, chronic overexpression of PI(4)5-K, which is a phospholipid

**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, rabbit; ra, rabbit; c, dog; x, Xenopus laevis.
a signaling cascade involving Rho-kinase, PI(4,5)-K, and its product, PI(3,4,5)-P3. We speculate that a sustained increase in PI(3,4,5)-P3 levels increases membrane ENaC through a mechanism independent of direct phosphatidylinositide binding to the channel. This mechanism likely is a more generalized trafficking response to chronic changes in PI(3,4,5)-P3. The physiological importance of PI(3,4,5)-P3 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 PI(3,4,5)-P3 levels. In contrast, increases in ENaC membrane levels parallel increases in PI(3,4,5)-P3 levels. Thus, as shown in the flow chart in Fig. 3, PI(3,4,5)-P3 may have multiple actions on ENaC to control channel activity.

**REGULATION OF ENaC BY PI(3,4,5)-P3**

**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. Paunescu and colleagues (55) demonstrated that aldosterone activates PI(3,4,5)-P3 to increase PI(3,4,5)-P3 levels in renal epithelia and that active PI(3,4,5)-P3 is required for aldosterone actions on Na+ transport mediated by ENaC. Several laboratories have subsequently confirmed the observations that aldosterone activates PI(3,4,5)-P3 in renal epithelia that active PI(3,4,5)-P3 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 PI(3,4,5)-P3 in epithelia and increase Na+ absorption mediated by ENaC in a PI(3,4,5)-P3-sensitive manner (20, 47, 48, 59). Importantly, insulin, while targeting basolateral receptors, quickly increases apical membrane PI(3,4,5)-P3 levels to affect activity of ENaC in this latter membrane (5). Active PI(3,4,5)-P3 also plays a role in setting basal ENaC activity in the absence of hormone stimulation (4, 55, 59). These findings suggest that active PI(3,4,5)-P3 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 PI(3,4,5)-P3 with ENaC in CHO cells increased channel activity (79, 86, 87). Similarly, addition of membrane-permeant PI(3,4,5)-P3 increased Na+ absorption across renal epithelia, and overexpression of a PI(3,4,5)-P3-phosphatase, phosphatase and TENsin homolog deleted on chromosome 10, attenuated the natriuretic effects of insulin and exogenous PI(3,4,5)-P3 (47). Activation of PI(3,4,5)-P3, moreover, with H2O2 provoked a rise in Na+ transport (48). Thus PI(3,4,5)-P3 activity is intimately tied to ENaC activity.

The mechanism by which aldosterone activates PI(3,4,5)-P3 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. PI(3,4,5)-P3 is a well-documented first effector of Ras (2, 60). In a recent publication (86), we argued that activation of PI(3,4,5)-P3 was a consequence of increased K-Ras expression in response to aldosterone.

Active PI(3,4,5)-P3 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 PI(3,4,5)-P3, PI(3,4,5)-P3, affects ENaC membrane levels by both a direct mechanism involving physical association of the phosphatidylinositide 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 PI(3,4,5)-P3 is a direct consequence of the physical association of PI(3,4,5)-P3 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 PI(3,4,5)-P3 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 Neddd4 family interact with the conserved PY motifs in the cytosolic COOH-terminal domains of ENaC subunits (1, 29, 72, 80, 81). This interaction facilitates ubiquitinylation 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 Neddd4 ubiquitin ligases (15, 70, 98). Association of Sgk with Neddd4 ubiquitin ligases leads to phosphorylation of the latter. This compromises the ability of Neddd4 ubiquitin ligases to bind and regulate ENaC. Thus activation of Sgk promotes retention of ENaC in the membrane. Sgk is a downstream effector of PI(3,4,5)-P3 sensitive to both PI(3,4,5)-P3 levels and phosphorylation by another PI(3,4,5)-P3 effector, PKD1 (22, 86, 90). Activation of PI(3,4,5)-P3 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 PI(3,4,5)-P3. The physiological importance of

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**Fig. 3.** PIP2 and PIP3 have both direct and indirect effects on ENaC open probability and membrane number. The flow chart shown here describes the effects of PIP2 and PIP3 on ENaC open probability (P0) and membrane levels (N). Up and down arrows indicate increases and decreases in phosphatidylinositide levels, and filled and dashed arrows indicate responses resulting from direct interaction of the phosphatidylinositide with the channel and those resulting from a signaling cascade that utilizes the phosphatidylinositide as a second messenger, respectively.
this PI3-K-sensitive mechanism controlling ENaC membrane levels is clear. Disruption of the FY 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 cells 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 NH₂ terminus of γ-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 β-ENaC. The importance of this similarity, as well as the exact function of this domain in γ-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 phosphatidylinositol products of PI3-K, PIP₁ and/or phosphatidylinositol-3,4-bisphosphate [PI(3,4)P₂], 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)P₂ levels, and ENaC activity in CHO cells (87). ENaC activity increased simultaneously with increases in membrane PIP₃/PI(3,4)P₂ 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)P₂ 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)P₂ 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 binding site in ENaC involves 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 phosphatidylinositide 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 phosphatidylinositide 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 phosphatidylinositol-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,

![Fig. 4](http://ajprenal.physiology.org/)
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 translocation 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 then global changes in membrane phosphatidylinositide 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 phosphatidylinositide 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 phosphatidylinositide 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 phosphatidylinositide levels in the luminal membrane and more specifically near the channel that are important for phosphatidylinositide 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 phosphatidylinositide 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 phosphatidylinositide turnover is present in many of the cases studied. Moreover, the tight temporal coupling of membrane phosphatidylinositide 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 phosphatidylinositides 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 phosphatidylinositides appears to develop more slowly. We excitedly await further determination of the physiological relevance of spatial and temporal control of cellular phosphatidylinosite levels with respect to control of ENaC activity.

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REFERENCES


