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Physiologic regulation of the epithelial Na^+ channel (ENaC) by phosphatidylinositides

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Abstract

Purpose of review—Epithelial Na^+ channel (ENaC) activity is limiting for Na^+ reabsorption in the distal nephron. Humans regulate blood pressure by fine-tuning Na^+ balance through control of ENaC. ENaC dysfunction is causative for some hypertensive and renal salt wasting diseases. Thus, it is critical to understand the cellular mechanisms controlling ENaC activity.

Recent findings—ENaC is sensitive to phosphatidylinositol 4,5-bisphosphate (PIP_2), the target of phospholipase C (PLC)-mediated metabolism, and phosphatidylinositiol 3,4,5-trisphosphate (PIP_3,), the product of phosphatidylinositide 3-OH kinase (PI3-K). PIP_2 is permissive for ENaC gating possibly interacting directly with the channel. Activation of distal nephron P2Y receptors tempers ENaC activity by promoting PIP_2 metabolism. This is important because gene deletion of P2Y2 receptors causes hypertension associated with hyperactive ENaC.

Aldosterone, the final hormone in a negative-feedback cascade activated by decreases in blood pressure, increases ENaC activity. PIP_3 sits at a critical bifurcation in the aldosterone-signaling cascade, increasing ENaC open probability and number. PIP_3-effectors mediate increases in ENaC number by suppressing channel retrieval. PIP_3 binds ENaC, at a site distinct form that important to PIP_2 regulation, to modulate directly open probability.

Summary—Phosphoinositides play key roles in physiologic control of ENaC and perhaps dysregulation plays a role in disease associated with abnormal renal Na^+ handling.

Keywords

PI3-K; purinergic receptor; aldosterone; PLC; hypertension

Introduction

The epithelial Na^+ channel (ENaC) functions as an effector for regulation of systemic and local fluid volume and electrolyte content (reviewed by [1, 2, 3]. Thus, this ion channel plays an important role in physiologic control of blood pressure and mucosal fluidity. Dysfunction and improper regulation of ENaC can be causative for disease associated with improper Na^+ handling, such as some forms of hypertension [4, 5, 6, 7].

Phosphoinositides serve as important second messengers in many intracellular signaling cascades. It is widely appreciated that in many instances phosphoinositides directly bind ion

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channel targets, including ENaC, to modulate channel gating and activity [8, 9, 10]. Disruption of phosphoinositide regulation of ion channels can lead to disease (e.g. Bartter's, Andersen's and long QT syndromes; [11, 12, 13, 14, 15, 16]. We discuss here advances in the understanding of phosphoinositide regulation of ENaC.

Physiologic regulation of ENaC by PIP_2

The activity of most ion channels sensitive to phosphoinositides, including the first channel identified to be directly regulated by PIP_2, K_{ATP} (Kir6.2; [17], rapidly decrease when excised from the cell membrane in an inside-out patch configuration. This hallmark termed "run-down" results, in part, from loss of PIP_2 [8, 9, 10].

Ma and colleagues [18] provided the first evidence that phosphoinositides directly modulate ENaC activity. This group showed that ENaC in excised, inside-out patches has characteristic run-down. Addition of exogenous PIP_2 to the intracellular face of ENaC countered run-down. Conversely, scavenging PIP_2 with an antibody and increasing PIP_2 metabolism in response to activating endogenous phospholipase C (PLC) accelerated ENaC run-down. These observations clearly established that ENaC, like K_{ATP}, is sensitive to PIP_2.

Several laboratories subsequently confirmed the relation between changes in membrane PIP_2 levels and ENaC activity with increases and decreases in PIP_2 rapidly causing corresponding increases and decreases in ENaC activity and Na^+ transport [19, 20, 21, 22, 23]. Figure 1 shows typical ENaC run-down in an excised, inside-out patch. Also shown in this figure are subsequent increases and decreases in ENaC activity in response to treatment with exogenous PIP_2 followed by scavenging PIP_2 with poly-L-lysine.

Regulation of ENaC by PIP_2 is physiologically important. Kunzelmann and colleagues [20] showed that stimulating G-protein coupled purinergic receptors inhibits amiloride-sensitive Na^+ absorption in airway and immortalized collecting duct epithelial cells by promoting PIP_2 metabolism. Amiloride is an open channel blocker of ENaC. We recently extended this initial observation by showing in mouse collecting duct epithelial cells that stimulating metabotropic purinergic receptor signaling decreases ENaC open probability via activating PLC and promoting apical membrane PIP_2 metabolism [22]. Thus, similar to other phosphoinositide-sensitive channels, PIP_2 modulates ENaC gating to affect open probability [22, 23].

Regulation of ENaC open probability by PIP_2 is rapid and can be dynamic [21, 22, 23]. Figure 2 shows the typical ENaC response to purinergic regulation via PLC-mediated PIP_2 metabolism in immortalized principal cells and an isolated, rat collecting duct preparation. Such regulation is particularly important when considering the recent findings that mice engineered to lack P2Y2 purinergic receptors have facilitated renal Na^+ reabsorption and are hypertensive as expected with hyperactive ENaC [24].

Similar to G-protein coupled receptors, signaling through receptor tyrosine kinases and phosphotyrosine phosphatases is capable of modulating ENaC activity by influencing membrane PIP_2 levels [21, 23]. Importantly, all studies agree that there is tight spatiotemporal coupling between the levels of PIP_2 in the membrane and ENaC activity/ open probability. Similar to that for other phosphoinositide-sensitive channels, this tight coupling is indicative of a possible direct effect of PIP_2 on ENaC (see below).

An observation providing additional support to the idea that PIP_2 modulation of ENaC is physiologically relevant is the recent finding that resting levels of PIP_2 in the apical membrane set basal ENaC activity [22]. This supports tonic regulation of ENaC by PIP_2. With such a setting, dynamic changes in membrane PIP_2 levels in response to signaling

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rapidly translate into changes in ENaC activity as is the case for decreases in ENaC activity in response to PIP_2 metabolism promoted by purinergic signaling and increases in ENaC activity in response to inhibiting PLC in unstimulated epithelial cells [22].

Mechanism of PIP_2 regulation of ENaC

Much experimental evidence, particularly the observation that exogenous PIP_2 prevents decreases in ENaC open probability in excised patches, suggests that PIP_2 regulation of ENaC is immediate meaning that the phosphoinositide likely binds the channel protein or a protein closely associated with the channel. This mechanism appears common to most phosphoinositide-sensitive channels with the channel proteins capable of interacting directly with regulatory phosphoinositides [8, 9, 10]. Supporting direct interaction between PIP_2 and ENaC are co-precipitation studies where channel subunits segregate with PIP_2 isolated with anti-PIP_2 antibody [19, 20]. We must guard, though, against over interpretation here. For in the absence of empirical biochemical evidence directly quantifying PIP_2 binding to purified channel protein or high resolution structural information, the current evidence that PIP_2 binds ENaC or, for the matter, any other channel proteins is only strongly circumstantial. This complicates a detailed understanding of molecular mechanism. The mechanism that we currently favor is that PIP_2 binding to ENaC counters negative regulation of open probability [23]; see below).

Regulation of ENaC by PIP_2 has many parallels with regulation of other ion channels, including Kir, 2-P domain and KCNQ K^+ channels and P/Q- and N-type Ca^{2+} channels, by this phosphoinositide (reviewed in [8, 9, 10, 25, 26]. Regulation affects channel gating and appears to be a direct consequence of phosphoinositide binding. Initial studies suggested that PIP_2, which is relatively abundant in the plasma membrane [27, 28], is permissive for ENaC activity (that is, function is dependent on the presence of this phosphoinositide) with decreases in this phosphoinositide causing decreases in ENaC activity. A permissive role for PIP_2 suggests that ENaC, similar to IRK and some KCNQ (e.g. KCNQ3) channels [29, 30], has a relatively high apparent binding affinity for this phosphoinositide. However, the most recent findings from collecting duct epithelial cells demonstrating tonic and dynamic regulation of ENaC by signaling pathways influencing PIP_2 levels support rather a signaling role (that is, when the phosphoinositide's changing abundance dynamically regulates function [22]). This would place ENaC into the group of phosphoinositide-sensitive channels, including GIRK and KCNQ2 & 4, with more moderate to low apparent PIP_2 binding affinities [29, 30].

While details about the possible molecular mechanism how PIP_2 increases ENaC open probability are debatable, progress has been made identifying the specific regions of ENaC necessary for a PIP_2 response. The three studies addressing this subject agree that the α-subunit of heterotrimeric (αβγ) ENaC has little role in transducing a PIP_2 response [19, 20, 23]. Rather the β- and possibly γ-subunits of heterotrimeric ENaC play a major role. Support for this position comes from co-precipitation studies where β- and γ-ENaC subunits are pulled-down by PIP_2 [19, 20]. Also in agreement are the findings of a recent mutagensis study demonstrating that deletion and charge neutralization of the extreme NH_2-terminus of β- and γ-ENaC subunits protect ENaC activity against decreases in PIP_2 [23]. This latter finding is interesting for disrupting these regions of ENaC had no effect on basal activity but did protect against decreases in activity due to PIP_2 metabolism. As mentioned above, this result led us to propose that PIP_2 relieves negative regulation of ENaC open probability. Although, the basis of this negative regulation currently is unknown, it might involve the NH_2-terminus of ENaC subunits with PIP_2 possibly immobilize a negative element in this region to counter repression of open probability.

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Functional PIP_2 binding sites have been proposed for several phosphoinositide-sensitive channels, including Kir and TRP channels, with a high resolution structure in the absence of PIP_2 available for these regions in Kir2.1 and 3.1 [29, 31, 32, 33, 34, 35]. A simplistic understanding is that these putative binding sites contain several well-conserved, positive charged residues that form a binding pocket/loop favoring electrostatic interactions between the polar head groups of the phosphoinositides within the inner leaflet of the plasma membrane and binding residues. The NH_2-terminal tails of ENaC subunits contain two tracts rich with conserved positive-charged residues: one at the extreme NH_2-terminus; and the other just proximal to the first transmembrane domain. The COOH-tail contains one such tract in the cystolic portion of the channel just following the second transmembrane domain. In a mutagensis study, we identified the regions in the extreme NH_2-termini of β- and γ-ENaC as being necessary for PIP_2 regulation of ENaC [23]. The conserved positive charged residues within this putative binding site were particularly important for neutralization of them was equivalent to deletion of the entire tract. Additional research is required to determine definitively whether these regions participate in a bona-fide PIP_2 binding site. In our study, the regions just proximal to the first transmembrane domains and just following the second transmembrane domains in β- and γ-ENaC where disqualified as having a role in PIP_2 regulation. In contrast to our study, the only other detailed investigation of potential PIP_2 binding sites within ENaC, identified the region just proximal to the first transmembrane domain in the NH_2-terminus of β-ENaC as being important [20]. The apparent discrepancy between these studies is yet to be resolved.

Physiologic regulation of ENaC by PIP_3

A physiological role for PI3-K and its product, PIP_3, in modulating ENaC activity is firmly established [36, 37, 38, 39, 40, 41, 42]. This phospholipid kinase is one downstream mediator of aldosterone action on the channel. The steroid hormone aldosterone increases ENaC activity, in part, by *trans*-activating serum and glucocorticoid-inducible kinase (Sgk) expression [44, 45]. Moreover, aldosterone increases PI3-K activity through a yet established mechanism [36, 37]. Sgk occupies a position in the PI3-K pathway homologous to that of Akt. In response to aldosterone, both the absolute and active levels of Sgk increase [41, 46]. PI3-K and the PIP_3-effector kinase PDK1 phosphorylate Sgk to activate it. The level of active ENaC in the plasma membrane is set, in part, by retrieval in response to channel modification by Nedd4 family ubiquitin ligases (reviewed by [42, 47]. Active Sgk phosphorylates Nedd4 ubiquitin ligases making them susceptible to sequestration away from ENaC by 14-3-3 chaperon proteins [48]. This effective uncoupling of Nedd4, which binds ENaC to modify it, from the channel facilitates ENaC activity by increasing its membrane half-life. Thus, PI3-K and PIP_3 signaling via Sgk repress ENaC retrieval to increase activity. This regulation is clearly important for naturally occurring variants in Sgk and Nedd4 family ubiquitin ligases that affect their ability to regulate ENaC have been linked to hypertension in man [49, 50, 51, 52]. Moreover, disruption of the PY motif in ENaC through which the channel interacts with Nedd4 ligases is the primary cause of the Mendelian form of hypertension termed Liddle's Syndrome [53, 54].

Several recent studies demonstrate that in addition to controlling the levels of ENaC in the plasma membrane, PI3-K and PIP_3 signaling also increase the open probability of ENaC (discussed directly below). Thus, PIP_3 signaling is capable of increasing ENaC activity via two mechanisms: increasing the number of functional channels in the membrane by repressing retrieval; and increasing the time channels within the membrane spend in the open state.

Addition of exogenous PIP_3 quickly activates ENaC in excised inside-out patches [18, 55]. Moreover, in outside-out patches, a setting where ENaC does not run-down, addition of

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exogenous PIP_3 increases ENaC activity above basal levels [23, 55, 56]. Figure 3 shows a representative experiment where ENaC open probability is increased by exogenous PIP_3 in an outside-out patch. This parallels findings where overexpression of active PI3-K and inhibition of this kinase increase and decrease ENaC open probability, respectively [55, 56]. In such experiments, changes in ENaC open probability and membrane PIP_3 levels follow identical time-courses indicating close spatiotemporal coupling between the phosphoinositide modulator and the channel [23, 55, 58]. These observations led to the hypothesis that like PIP_2, PIP_3 is also capable of exerting a direct effect on channel gating with this stimulatory action on open probability mediated by binding of the phosphoinostide to the channel or a protein closely associated with the channel.

The physiologic importance of PI3-K and PIP_3 to modulation of ENaC open probability in native epithelial cells was recently confirmed in an isolated collecting duct preparation [58]. Reminiscent of PIP_2 regulation, dynamic changes in ENaC open probability mirrored changes in apical PIP_3 levels in collecting duct principal cells [58]. Figure 4 shows in polarized epithelial cells that rapid decreases in ENaC open probability (4A) parallel decreases in apical membrane PIP_3 levels upon inhibition of PI3-K (4B). Again, manipulating PIP_3 signaling fast provokes changes in ENaC open probability. This mechanism for acute and dynamic regulation of ENaC by PIP_3 may be particularly relevant to modulation of the channel by aldosterone and other hormones that promote Na^+ retention, including insulin and IGF-I. For PI3-K signaling is required for the natriferic activity of these hormones and these hormones quickly increase PI3-K activity and apical PIP_3 levels [38, 41, 46, 58, 59, 60].

Mechanism of PIP_3 regulation of ENaC

The prediction of a physical association between ENaC and PIP_3 initially presented a conceptual challenge. For if, ENaC is sensitive to PIP_2 and PIP_2 is 100-1000 times more common in the plasma membrane than PIP_3 [27, 28], then how can a phosphoinositide binding site within ENaC ever be occupied by PIP_3? This conundrum, which intuitively did not jibe with the experimental evidence demonstrating PIP_3-specific activation of ENaC open probability even in the presence of overwhelming PIP_2 (see above), was solved by detailed investigation of potential phosphoinostide binding sites within ENaC.

Recall that the regions of ENaC important to PIP_2 regulation are the positive charged residues in the NH_2-termini of β- and γ-EN subunits. In two papers using a combination of mutagenesis, electrophysiology and biochemistry, we defined the regions of ENaC important to PIP_3 regulation to the cytosolic portions of the β- and γ- subunits just following the second transmembrane domains, which contain several positive charged residues [23, 56]. Deletion and charge neutralization of these residues abolished PIP_3 stimulation. In addition, disrupting these tracts prevented co-precipitation of channel subunits with PIP_3 [56]. It is interesting that, as for PIP_2 regulation, α-ENaC subunits again appear to play little role in regulation by PIP_3.

If the prospect that ENaC can bind PIP_3 is correct, which is consistent with all current findings, then the PIP_3 binding site is likely not well occupied at rest and available for dynamic regulation of the channel. In keeping with the idea that phosphoinositides physically interact with ENaC to directly influence channel gating, then the simplest interpretation of the above observations is that there are two distinct phosphatidylinositide binding sites within ENaC with one preferring PIP_3 and the other PIP_2.

The emerging understanding regarding putative phosphatidylinositide binding sites within ENaC is that they are in cytosolic regions of the channel often close to the gate and that the conserved positive-charged residues within these domains play an important role in

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phosphoinositide binding. This understanding is similar to that for putative phosphoinositide binding sites in other ion channels, including TRP and Kir, where direct phosphoinositide binding is believed to affect channel gating [29, 31, 32, 33, 34].

It is not clear yet what provides selectivity to phosphoinositide binding sites within ion channels; however, alanine substitution of the conserved negative-charged and bulky residues within the putative PIP_3 binding site in γ-ENaC enhances basal activity and responses to PI3-K signaling [56]. These findings suggest that these residues, in addition to the conserved positive charged residues, may influence binding affinity and selectivity. Non-charged and negative-charged residues in the putative binding sites of other phosphatidylinositide-sensitive channels are thought to play a similar role [29, 61].

Conclusion

Much progress has been made recently in understanding phosphoinositide regulation of ENaC. The cell model shown in figure 5 summarizes contemporary thinking about how signaling pathways having phosphoinositide second messengers are coupled to ENaC. An emerging theme is that, in some instances, these signaling molecules directly bind the channel to influence open probability. This mechanism of regulation is dynamic enabling the channel and thus, the epithelial cell to respond rapidly and properly to locale and systemic cues reflecting a need for changes in Na^+ balance. Much evidence points to an important physiological role for phosphoinositde regulation of ENaC. Perhaps, dysfunction of this regulation contributes to pathology in man as attested to by the hypertensive state of P2Y2 knock-out mice [24].

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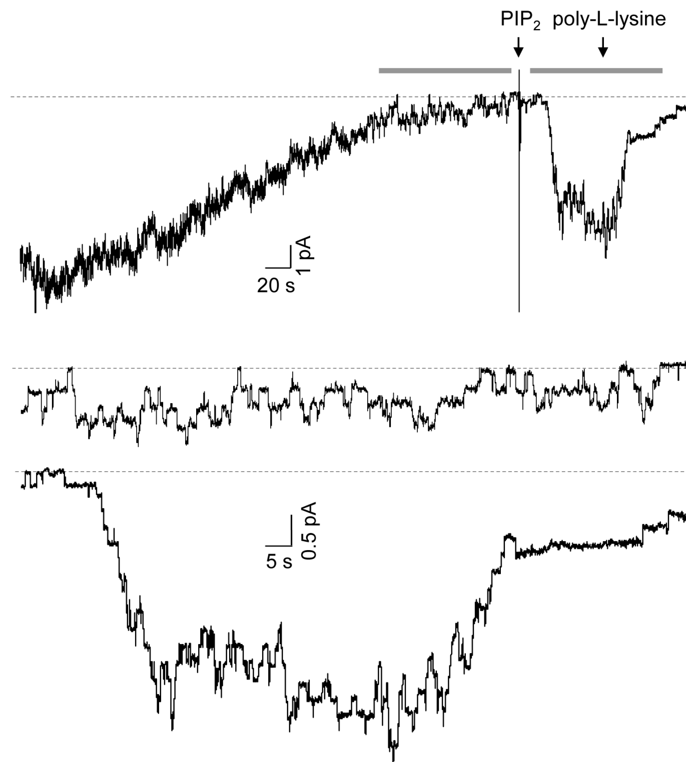

Figure 1. PIP_2 activates ENaC

Current trace of ENaC expressed in a CHO in an excised, inside-out patch. The patched membrane was clamped to 0 mV. Inward Na^+ currents are downward with the dashed gray line noting the closed state. Over the course of this experiment, ENaC activity ran-down and was then re-activated by addition of 30 μM PIP_2 to the bath solution (noted with first arrow). ENaC activity was subsequently decreased upon addition of the PIP_2 scavenger poly-L-lysine (20 μg/ml; noted with second arrow). The complete experiment is shown in the top trace with the middle and bottom traces showing the areas under the gray bars before and after PIP_2 addition at expanded time and amplitude scales. Figure adapted from [25].

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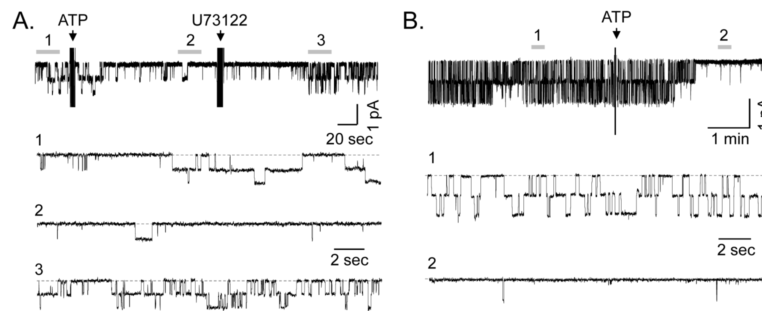

Figure 2. ATP inhibits ENaC activity in polarized renal epithelial cells via activation of PLC
A. Representative current traces of ENaC in a cell-attached patch containing before and after treatment with ATP in the absence and presence of the PLC inhibitor U73122. Patches made on the apical membrane of polarized mpkCCDc14 cells avidly reabsorbing Na^+ [62]. Inward Li^+ current is downward. This patch was held at a test potential of - V_p= – 40 mV. Areas (1), (2), and (3) are shown below at an expanded time scale. Dashed line notes the closed state. Figure adapated from [22]. **B.** Current traces of ENaC before and after addition of ATP to a cell-attached patch made on a principal cell from a collecting duct freshly isolated from a salt restricted rat. (see [58] for a more complete description of this preparation.) Traces before and after ATP are shown below at expanded time scales. This patch was clamped to $-V_p$ = – 60 mV with Li^+ as the permeant cation in the pippette solution. Inward current is downwards.

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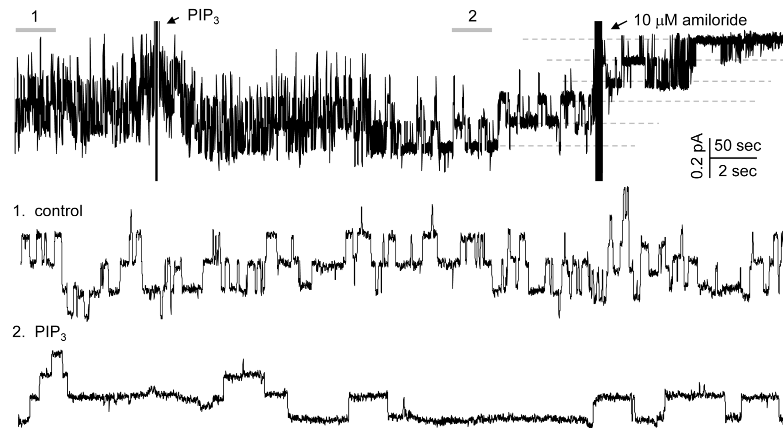

Figure 3. PIP$_3$ activates ENaC

Shown is a representative current trace from an excised, outside-out patch (V_p = 0 mV) formed on a CHO cell expressing ENaC before and after addition of 20 μM exogenous diC8 PIP$_3$. PIP$_3$ added to the bathing solution in the presence of histone H1 carrier. Amiloride subsequently added to the bath solution towards the end of the experiment. This representative patch contains, at least, five ENaC. Shown at top is a continuous trace. Shown below at an expanded time-scale are regions of the trace before (1. control; middle) and after (2. PIP$_3$; bottom) addition of exogenous phosphoinositide. Inward Na^+ current is downwards.

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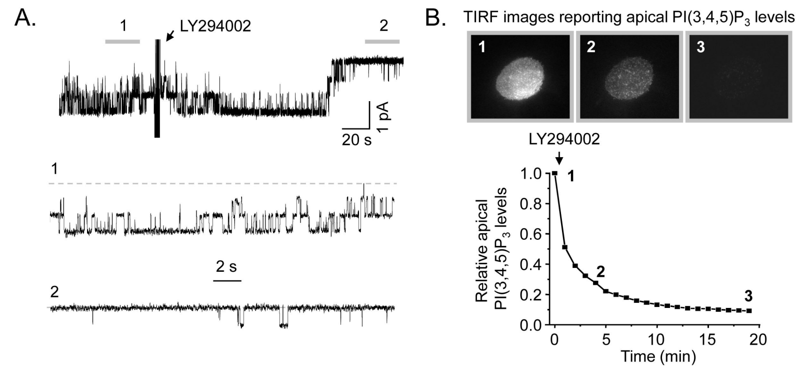

Figure 4. ENaC open probability parallels changes in apical PIP_3 levels in collecting duct principal cells

A. Continuous current trace of ENaC before and after inhibition of PI3-K with LY294002 in a cell-attached patch made on a principal cell from a collecting duct freshly isolated from a salt restricted rat. Traces before and after inhibition of PI3-K are shown below at expanded time scales. This patch was clamped to $-V_p = -60$ mV with Li^+ as the permeant cation in the pippette solution. Inward current is downwards. **B.** Fluorescence micrographs showing emissions from the PIP_3 reporter GFP-AktPH [63, 55] in the apical membrane of a principal cell (mpkCCDc14) within a tight monolayer before and after inhibiting PI3-K. Emissions at the apical membrane were opitically isolated with total internal reflection fluorescence microscopy [64, 65, 55]. The diary plot below shows the relative decrease in apical PIP_3 levels in this cell over time following inhibition of PI3-K. Figure modified from that presented in [26].

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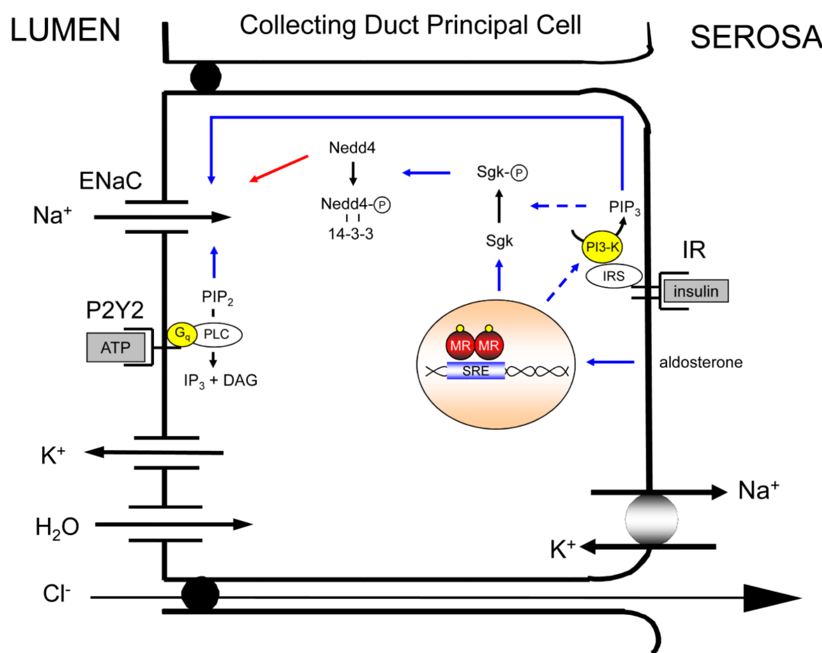

Figure 5. Physiological regulation of ENaC by signaling pathways using phosphoinositide second messengers

This cell model summarizes contemporary thinking about how signaling pathways having phosphoinositide second messengers regulate ENaC. Blue and red arrows indicate positive and negative regulation, respectively. Dashed lines indicate multiple steps, which are not explicitly shown in this figure, or steps where the mechanism remains unknown. The abbreviations IP_3, DAG, G_q and IRS have their usual meaning. IR, MR and SRE are abbreviations for insulin receptor, mineralocorticoid receptor and steroid-response element, respectively. All other abbreviations mentioned in text.