Acute Regulation of Epithelial Sodium Channel by Anionic Phospholipids

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Anionic phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol 3,4,5-trisphosphate (PIP3) are normally located in the inner leaflet of the plasma membrane, where these anionic phospholipids can regulate transmembrane proteins, including ion channels and transporters. Recent work has demonstrated that (1) ATP inhibits the renal epithelial sodium channel (ENaC) via a phospholipase C–dependent pathway that reduces PIP2, (2) aldosterone stimulates ENaC via phosphoinositide 3-kinase, and (3) PIP2 and PIP3 regulate ENaC. Several lines of evidence show that ATP stimulation of purinergic P2Y receptors hydrolyzes PIP2 and that aldosterone stimulation of steroid receptors induces PIP3 formation. These studies together suggest that one primary mechanism for regulating ENaC is by alteration of anionic phospholipids and that the receptor-mediated and hormonal regulation of ENaC works through a variety of signaling pathways, but many of these pathways finally alter ENaC activity by regulating the formation or degradation of anionic phospholipids. Therefore, changes in the concentration of PIP2 and PIP3 are hypothesized to participate in the regulation of ENaC by purinergic and corticoid receptors. The underlying mechanism may be associated with a physical interaction of the positively charged cytoplasmic domains of the β- and γ-ENaC with the negatively charged membrane phospholipids. The exact nature of this interaction will require further investigation.


The phospholipid compositions of the two leaflets of the lipid bilayer that forms the plasma membrane are strikingly different. Anionic phospholipids are normally located in the inner leaflet to form a negatively charged surface. However, whether the phospholipid asymmetry affects the function of transmembrane proteins remains largely unknown. Recent studies have shown that one of the anionic phospholipids, phosphatidylinositol 4,5-bisphosphate (PIP2), regulates Na+/Ca2+ exchangers and inward rectifier potassium channels (1–4). Besides PIP2, phosphatidylinositol 3,4,5-trisphosphate (PIP3) regulates ATP-sensitive potassium (KATP) channels (5,6). A model for the regulation of KATP channels by anionic phospholipids has been proposed; the negatively charged head group of PIP2 or PIP3 locks the positively charged carboxyl terminus of KATP channels in an open conformation and prevents ATP binding to the cytosolic terminus (7). These recent studies suggest that anionic phospholipids may interact with the positively charged cytoplasmic termini of other ion channels and transporters. In this review, we focus primarily on the mechanism by which changes in cellular anionic phospholipids such as PIP2 and PIP3 regulate the renal epithelial sodium channel (ENaC) and then speculate on a possible underlying mechanism.

Stimulation of Purinergic P2Y Receptors Decreases Membrane PIP2 and ENaC Activity

It is known that ATP binds to the purinergic P2Y receptor family, G protein–coupled receptors that activate phospholipase C (PLC) to hydrolyze PIP2, resulting in a decrease in PIP2 concentration in the inner leaflet of the plasma membrane. P2Y receptors, particularly the P2Y2 receptor, are expressed in renal tissues (8–11). ATP binding to the P2Y2 receptor inhibits Na+ absorption in mouse cortical collecting duct principal cells (10). Our recent studies show that ATP inhibits the activity of ENaC via a PLC-dependent pathway in A6 distal nephron cells (12). These studies suggest that ENaC is regulated by purinergic P2Y receptors. The traditional signaling pathway after G protein–coupled receptor activation of PLC involves the breakdown of PIP2 to form inositol 1,4,5-trisphosphate (IP3) and diacylglycerol, which together lead to increases in intracellular Ca2+ and activation of protein kinase C (PKC). One or the other of these then is thought to produce the final effect on ENaC activity after receptor stimulation. Thus, PIP2 is often considered to be nothing more than a substrate for PLC with the metabolic products being the important signaling molecules.

Although there is little question that activation of PKC can alter...
ENaC activity (13–17), our work has shown that ENaC is also directly regulated by PIP₂ (18,19). We demonstrated that PIP₂ added to the cytosolic surface maintains ENaC activity in inside-out patches. In contrast, both sequestration of endogenous PIP₂ with anti-PIP₂ antibody and hydrolysis of PIP₂ after activation of endogenous PLC or addition of exogenous PLC reduced channel activity. When expressed in *Xenopus* oocytes, ENaC is stimulated by cytosolic injection of PIP₂ (18). These observations suggest an additional and alternative mechanism for P2Y₂ receptor regulation of ENaC in which activation of PLC reduces PIP₂ concentration and the reduction in PIP₂ (rather than the production of IP₃ and diacylglycerol) causes the reduction in ENaC activity. Therefore, a decrease in PIP₂ concentration induced by purinergic receptor activation may account for some of the inhibition of ENaC by ATP. As mentioned above, there is ample evidence that when PKC is activated, ENaC is inhibited, and when PKC is inhibited, ENaC is activated. However, the phosphorylation target for PKC has remained elusive. The best evidence suggests that Ca²⁺ inhibits its ENaC in intact cells but does not inhibit ENaC in excised inside-out patches (17) and that PKC inhibits ENaC in A6 cells (17) but does not phosphorylate ENaC (20). Furthermore, at least in oocytes, when PKC is activated with phorbol ester, membrane levels of PIP₃ decrease and PIP₃-dependent inward rectifier potassium channel activity is reduced (21,22). These results suggest that PKC may also regulate ENaC by alteration of PIP₂, but further investigation will be required to determine whether this is the mechanism for PKC inhibition of ENaC.

**Stimulation of Steroid Receptors Increases Membrane PIP₃ and ENaC Activity**

Besides PIP₂, PIP₃ regulates Kᵦᵦᵦ channels (5,6) and other inward rectifier potassium channels (23–25). However, its role has not attracted as much attention because, under normal conditions, basal PIP₂ levels are very low in cells, even though it is well known that PIP₃ can be generated from PIP₂ by activation of phosphotyidylinositol-3-kinase (PI-3-K). Recent studies suggest that both aldosterone and insulin enhance Na⁺ transport by activating PI-3-K in A6 cells (26–28) and that the concentration of PIP₃ in A6 cells is elevated in response to aldosterone (26). Recent studies have also shown that not only PIP₂ but also other anionic phospholipids, including PIP₃, acutely regulate ENaC activity in A6 cells (18,29). Aldosterone stimulates ENaC in two phases: an acute phase, which is associated with the elevation of channel open probability, and a chronic phase, which is possibly related to an increase in cell surface expression of ENaC (30–35). Because PIP₃ acutely increases ENaC open probability (29), PIP₃ formation may mediate some or all of the acute effect of aldosterone on ENaC open probability.

However, anionic phospholipids, particularly inositol lipids phosphorylated at the 3 position, are also involved in vesicle trafficking to the plasma membrane (36–39). For example, a PI-3-K inhibitor (wortmannin) and dominant-negative PI-3-K block the insulin-mediated translocation of GLUT4 glucose transporter to the plasma membrane (40,41). Therefore, besides acutely increasing ENaC open probability, PIP₃ may mediate at least part of the chronic effect of aldosterone on ENaC cell surface expression. Because insulin also elevates ENaC activity via PI-3-K (28,42), PIP₃ likely also mediates the regulation of ENaC by insulin (43,44).

**Are There Any Consensus Sequences for PIP₂ and PIP₃ Binding?**

Almost a decade has passed since PIP₂ was found to regulate membrane proteins such as the Na/Ca exchanger and inward rectifier potassium channels. However, no simple consensus sequence for PIP₂ binding has been identified. Although pleck-strin homology (PH) domains have been described in >100 proteins, a careful BLAST search reveals that there is no strict homology between these domains. This may not be surprising because x-ray crystallographic studies suggest that a three-dimensional structure is required for specific recognition of phosphoinositides (45). Despite that phosphoinositide binding sites have no universal consensus sequence, one common feature of these PH domains is that all of them contain significant numbers of positively charged lysines or arginines, and it is generally accepted that PIP₂ or PIP₃ binding is closely associated with these positively charged lysine and arginine residues. Therefore, any proteins that contain repeating lysine- or arginine-rich motifs may bind phosphoinositides and be regulated by PIP₂ and PIP₃ even though there is no common consensus sequence for the binding domains (45).

Despite that most of the domains were found to bind phospholipids with high affinity but low selectivity (46), two exceptions are the PH domain of PLC₆₁ (PLC₆₁-PH domain) and the PH domain of the general receptor for phosphoinositides-1 (GRP1-PH domain), which respectively bind to PIP₂ and PIP₃ with high affinity and high selectivity. The GRP1-PH domain selects PIP₃ over PIP₂ by >100-fold (37,47). To determine the molecular basis that accounts for the different binding affinity, the sequences of PLC₆₁-PH (Figure 1A) and GRP1-PH (Figure 1B) domains were compared as shown in Figure 1. By comparing the positions of positively charged lysine (K) and arginine (R) between the PLC₆₁-PH domain and the GRP1-PH domain, it seems that positively charged K and R in the PLC₆₁-PH domain are usually separated by other amino acids. Conversely, positively charged K and R in the GRP1-PH domain are close to each other. Using synthesized peptides, it has been shown that insertion of a K between two Ks abolishes PIP₂ binding (48). Previous crystallography studies also suggest that a specific three-dimensional structure is required for selective phosphoinositol binding (45,49,50). However, whether geometric localization of positively charged amino acids is important for the selectivity of PIP₂ and PIP₃ binding is not completely clear.

**Cytoplasmic Domains of β- and γ-ENaC Contain Possible PIP₂ and PIP₃ Binding Motifs**

ENaC consists of three subunits designated α, β, and γ (51). By analyzing the sequences of cytoplasmic domains of ENaC, several positively charged lysine- or arginine-rich motifs are consistently found in the cytoplasmic domains (especially N-termini, as shown in Figure 2) of β- and γ-ENaC among all species including human, rat, mouse, and *Xenopus*. It is known
that the C-termini of \(\beta\)- and \(\gamma\)-ENaC play an important role in regulating ENaC activity. Recent studies have also shown that deletion of the cytoplasmic N-termini of \(\beta\)- (\(\Delta 2\) to 49) and \(\gamma\)-ENaC (\(\Delta 2\) to 53) but not the homologous N-terminal section of \(\alpha\)-ENaC (\(\Delta 2\) to 46) dramatically reduces ENaC activity (52), suggesting that the N-termini of \(\beta\)- and \(\gamma\)-ENaC also regulate ENaC activity. Therefore, PIP\(_2\) and PIP\(_3\) may regulate ENaC by interacting with its cytoplasmic domains at arginine- or lysine-rich regions and that these lysine- or arginine-rich motifs may represent the specific binding sites for both PIP\(_2\) and PIP\(_3\). Possible Role of PIP\(_2\) and PIP\(_3\) in Receptor-Mediated ENaC Regulation

Many hormones and transmitter agents alter ENaC activity in renal epithelial cells. These include such diverse agents as adenosine (53), dopamine (54), angiotensin II (55), insulin (56), prostaglandin E2 (57), purinergic agents (12), and aldosterone (31). Anionic phospholipids also regulate ENaC open probability (18,19). Any agent that regulates the membrane levels (by degradation or synthesis) of these lipids should regulate ENaC. Any anionic phospholipid in theory can produce this regulation, but the naturally occurring lipids PIP\(_2\) and PIP\(_3\) seem to be the most important. Because the production and degradation of each lipid are under the control of separate enzymes, they can be differentially regulated to produce varying physiologic responses. In this review, we have focused on two specific examples: One is related to a decrease in PIP\(_2\) caused by P2Y receptor–induced activation of PLC; the other is associated with an increase in PIP\(_3\) caused by steroid receptor–induced activation of PI 3-K.

Previous studies have shown that the PH domain of PLC\(\delta_1\) binds to both PIP\(_2\) and IP\(_3\) (50,58) and that the level of PIP\(_2\) in the plasma membrane and the production of IP\(_3\) can be visualized by the green fluorescent protein–tagged PH domain of PLC\(\alpha_1\) (59). Using this method, it was shown recently that PIP\(_2\) hydrolysis mediates EGF-induced inhibition of ENaC (60). We recently demonstrated that a decrease in membrane PIP\(_2\) accounts for ENaC inhibition induced by both purinergic receptor activation and ionomycin-induced elevation of intracellular calcium (61). Our studies have shown that PIP\(_2\) binds to both \(\beta\)- and \(\gamma\)-ENaC subunits (19), even though the binding sites for PIP\(_2\) have not been identified yet. As a hypothesis, we propose a working model in which ATP causes a loss of negative charges (PIP\(_2\)) on the inner surface of plasma membrane and thereby releases the positively charged cytoplasmic domains that lead to decreases in ENaC open probability, as shown in the top part of Figure 3.

It has also been shown that PIP\(_3\) in the cells can be selectively labeled by a specific green fluorescent protein–fused, GRP1-PH domain. Using this method, recent studies have shown that there is an obvious elevation of apical membrane PIP\(_3\) in response to insulin (43). We recently demonstrated that both PIP\(_3\) and the N-terminal tail of \(\gamma\)-ENaC are required for aldosterone-induced ENaC activity and trafficking (62). However, whether

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**Figure 1.** Amino acid sequences of phospholipase C \(\delta_1\) pleckstrin homology (PLC-\(\delta_1\)-PH) domain (A) and general receptor for phosphoinositides-1 PH (GRP1-PH) domain (B). Under physiologic conditions (pH 7.4), K and R residues contain positive charges, whereas D and E residues contain negative charges. Both positive and negative charges were marked under each amino acid. Putative phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) and phosphatidylinositol 3,4,5-trisphosphate (PIP\(_3\)) binding motifs are shown in bold.

**Figure 2.** Amino acid sequences of the N-termini of \(\beta\)-subunit (A) and \(\gamma\)-subunit (B) of epithelial sodium channel (ENaC). Under physiologic conditions (pH 7.4), K and R residues contain positive charges, whereas D and E residues contain negative charges. Both positive and negative charges were marked under each amino acid. Possible PIP\(_2\) and PIP\(_3\) binding motifs are shown in bold.
Figure 3. Working models for ENaC regulation by either ATP via a decrease in membrane PIP2 or aldosterone via an increase in membrane PIP3. Under resting conditions, the positively charged regions of the cytoplasmic termini of α- and β-ENaC are held in a certain position by the negatively charged PIP2 located in the inner leaflet of plasma membrane (top left). When luminal ATP is elevated, the concentration of PIP2 is decreased as a result of the activation of PLC via P2Y receptors. Loss of PIP2 “unlocks” the cytoplasmic termini to release them from the inner surface of plasma membrane, subsequently leading to the decreased ENaC open probability (top right). Aldosterone elevates the concentration of PIP3 by activating of phosphotidylinositol-3-kinas (PI 3-K). PIP3 stimulates ENaC activity by further “locking” of the cytoplasmic termini to the inner surface of plasma membrane because of one more negative charges (middle right). In the absence of aldosterone, the inner leaflet of the plasma membrane contains PIP2 rather than PIP3 (bottom left). In the presence of aldosterone, PIP3 is produced and somehow stimulates ENaC surface expression. Illustration by Josh Gramling—Gramling Medical Illustration.
PIP₃ interacts with the N-terminal tail of γ-ENaC remains to be determined. For this model to work, PIP₃ would need to be >10-fold more effective than PIP₂ because PIP₃ concentration, even after stimulation, is only approximately 10% of PIP₂ (24,25). Therefore, we suggest that the N-terminal tail of γ-ENaC contains a specific PIP₃ binding motif. We hypothesize a working model that aldosterone-induced synthesis of PIP₃ seems to be important both for acutely elevating ENaC open probability (Figure 3, top) and for increasing ENaC cell surface expression (Figure 3, bottom).

Conclusion
The purpose of this review is to provide a new explanation for the regulation of ENaC by purinergic and corticoid receptors. In particular, the concept of interaction of cytoplasmic domains of ENaC with anionic phospholipids suggests a novel mechanism by which ENaC could be regulated by the lipid composition of the inner leaflet of the plasma membrane.

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