ISSN 1990-7478, Biochemistry (Moscow) Supplement Series A: Membrane and Cell Biology, 2009, Vol. 3, No. 3, pp. 261–274. © Pleiades Publishing, Ltd., 2009.
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Regulation of the Epithelial Sodium Channels (ENaC) by Small G Proteins and Phosphatidylinositides[1]

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Received February 19, 2009

Abstract—The epithelial Na^+ channel (ENaC) plays a central role in control of epithelial surface hydration and vascular volume. ENaC activity in these epithelia is limiting for sodium reabsorption. Abnormalities in ENaC function have been linked to disorders of total body Na^+ homeostasis, blood volume, blood pressure, and lung fluid balance. Recently, ion channels were recognized as physiologically important effectors of small GTP-binding proteins and phosphatidylinositides. We review here recent findings relevant to regulation of ENaC by small G proteins and phosphatidylinositides.

Key words: epithelial sodium channel, ENaC, Ras, Rho, Rab, phosphatidylinositide-3,4,5-triphosphate ($PI(3,4,5)P_3$), phosphatidylinositide-4,5-bisphosphate ($PI(4,5)P_2$).

DOI: 10.1134/S1990747809030040

The maintenance of water–electrolyte balance is the main function of the mammalian excretory system. Ion and water excretion is finely regulated by different endocrine factors. Sodium reabsorption in the kidneys plays a central role in the salt homeostasis support and determines fluid volume in circulation and the whole organism. Epithelial sodium channel (ENaC) activity is a main limiting factor of the sodium absorption through epithelial layer in distal nephrone [1]. This channel is localized in the apical membrane of epithelial cells in kidney cortical collecting ducts, lungs and colon. The physiological role of this channel in system blood pressure regulation may be demonstrated by inheritable forms of hypertension which caused by ENaC hyperactivity like Liddle's syndrome. Indeed, mutations in genes encoding ENaC subunits caused a loss of the channel function and lead to pseudohypoaldosteronism type I (PHA-I) which is characterized by hypotension, hyperkalemia and salt wasting [2, 3].

ENaC is a highly selective sodium ion heteromeric channel consisting of α, β, and γ subunits [4]. All three subunits in 1:1:1 ratio are necessary for functional channel formation [5, 6]. The subunits contain short intracellular NH_2- and COOH- (~50–100 amino acid residues) termini divided by two transmembrane (TM1 and TM2) and one extracellular (~450 amino acid residues) domains [7]. By analyzing the sequences of cytoplasmic domains of ENaC subunits, several positively charged lysine- or arginine-rich motifs are consistently found in both N- and C-termini of β- and γ-ENaCs among all species including human, rat, mouse, and frogs. Both the C-termini and the N-termini of β- and γ-ENaCs play an important role in regulation of ENaC activity. Mutations in the C-terminus of β- or γ-ENaC elevates ENaC activity and cell surface expression and accounts for some forms of inherited hypertension or Liddle's syndrome [2, 8, 9].

To maintain Na^+ homeostasis, epithelial Na^+ absorption via ENaC must be tightly regulated. The rate of Na^+ absorption varies widely to respond to conditions of Na^+ deprivation and Na^+ excess. ENaC activity, like that of other ion channels, can be regulated by two fundamental mechanisms: changes in channel gating (P_o) or changes in the number of channels at the cell surface [10, 11]. Diverse signaling endocrine factors play a role in regulating ENaC cellular localization and activity. Aldosterone is one of the most important hormones regulating discretionary Na^+ reabsorption. The renal distal nephron principal cells are one of the primary targets for aldosterone. Here, aldosterone increases ENaC activity resulting in increased Na^+-dependent fluid reabsorption. At present, aldosterone has been found to upregulate the expression of several targets including SGK, GILZ, WNK, PI3-K, Usp2-45, and K-Ras [12–15]. Figure 1 describes two ways of Na^+ reabsorption regulation through epithelial cells: 1) channel open probability control (P_o), 2) stimulation of channel integration with plasma membrane or attenuation of channel internalization. Moreover, recent findings indicate that ENaC is activated by the proteolytic release of inhibitory peptides from the α- and γ subunits [16–19]. Thus, endo-, para- and autocrine factors recruit num-

[1] The article was translated by the authors.
[2] Equal contribution.

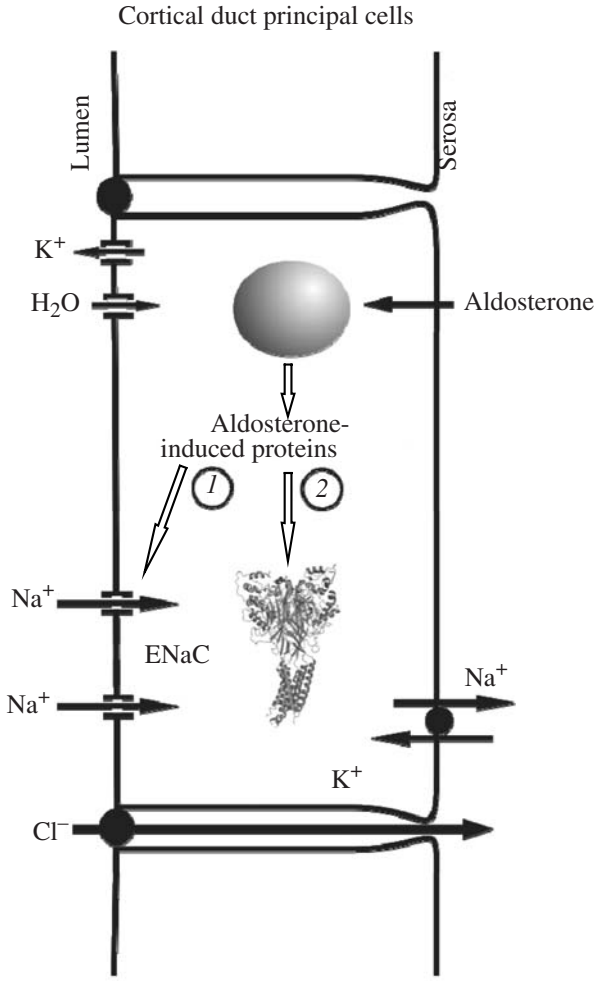

Fig. 1. Model of regulation of Na^+ reabsorption through the epithelial cells. There are two possible mechanisms of modulation of ENaC activity: 1) control of open probability (P_o) of functional channels presented on the cell surface – effect on the ENaC gating mechanisms, 2) promotion of channel insertion into plasma membrane or slowing of ENaC internalization.

bers of intracellular signal pathways for the ENaC-dependent sodium reabsorption in distal nephron.

REGULATION OF ION CHANNELS BY SMALL G PROTEINS

G proteins are members of a superfamily of GTPase-binding proteins. G proteins serve as GTP-dependent switches to control the activity of effector proteins and subsequently initiate associated signaling cascades. G proteins consist of two main groups – heterotrimeric and small G proteins. Both of them are involved in signal transduction. The heterotrimeric G protein consists of an α-subunit that binds and hydrolyzes GTP as well as of a β- and a γ-subunit that form an undissociable complex. Activation of heterotrimeric G proteins induces dissociation of subunits to α-GTP and βγ-dimer. Unique for each G protein, α-subunit determines specificity of interaction with receptor and effector. Small G proteins are monomeric GTPases with molecular masses ranging from 20 to 25 kDa. Their polypeptide chain is a homolog of α-subunit of heterotrimeric G proteins. G proteins have high sequence homology and share several conserved domains, including consensus amino acid sequences responsible for interaction with GDP and GTP, and a region interacting with downstream effectors. More than 100 small G proteins have been identified in eukaryotes [20]. The members of this superfamily are structurally classified into at least 5 families: 1) Ras, 2) Rho, 3) Rab, 4) Sar1/Arf, and 5) Ran. Sometimes, this group of proteins is termed the Ras superfamily in reference to one of the first proteins belonging to the small G proteins. However, now this classification is no longer used.

Most small G proteins are localized either in the cytosol or at plasma and intracellular membranes. Proteins can be in stable equilibrium between them [21]. Recently Heo and colleagues [22] have tested intracellular distribution of fluorescent protein-conjugated small G proteins in mammalian cells. Out of 125 small GTPases that were investigated, 48 were fully or partially localized to the plasma membrane [22].

Members of this superfamily are involved in regulation of wide variety cellular processes, including gene expression, cytoskeleton reorganization, cellular traffic, proliferation, and differentiation. Moreover, small G proteins play key roles in ion channels regulation [23]. Regulation of ion channels by heterotrimeric GTPases has been the focus of several recent reviews. However, regulation of ion channels by small monomeric G proteins has been reviewed less comprehensively.

Heterotrimeric G proteins control a huge variety of ion channels via signaling cascades, inducing stimulation or inhibition of phosphorylation, increase intracellular Ca^{2+} or ATP, etc. In some cases activated subunits of heterotrimeric G proteins can directly bind ion channels, modulating channel activity [24, 25].

Evidence that ion channels are final effectors of not only heterotrimeric G proteins but also small G proteins has accumulated for the last decade [26]. Small G proteins can increase or decrease activity of ion channels. For example, HA-Ras via mitogen-activated protein kinase (MAPK) signaling decreases the activity of inward rectifier K^+ channel 1 (IRK1) by promoting retrieval of the channel from the plasma membrane [27, 28] but increases the activity of T-type Ca^{2+} channels [29]. In some cases, small GTPases interact directly with ion channels to elicit regulation, and in others, regulation is mediated by intermediary signaling proteins. For example, members of the RGK family of small GTPases form a regulatory complex with the L-type Ca^{2+} channel β-subunit and decrease number of channels on the plasma membrane [30–32]. In contrast,

K-Ras increases the ENaC activity via PI3-kinase signaling.

Ion channels can be final effectors of several signaling cascades initiated by various G proteins. In this case, the effects of these signaling cascades can be opposite. For example, Rac1 and RhoA mediate opposing hormonal regulation of ERG potassium channels. Rho inhibits this channel via a protein serine/threonine (S/T) kinase, whereas Rac stimulates the channel via a protein S/T phosphatase [33]. Recently, it was shown that, the S/T phosphatase PP5 acts as a direct molecular effector for activated Rac [34]. Although Rho and Rac signaling pathways interact at many levels, Rac-dependent stimulation of PP5 provides a direct molecular mechanism for the antagonism of Rho-dependent signaling. In addition, Rho small G proteins modulate potassium channels Kv1.2, calcium and nonselective cation channels [34–38].

Small G proteins can control activity of the ion channels both by regulation of the number of channels on the cell surface and by modulation channel P_o [26]. In this case the number of the channels can be changed both via increase/decrease of channel trafficking to the plasma membrane and via promotion/inhibition of channel retrieval from the plasma membrane.

Recent findings show that Rab proteins play a key role in the regulation of a variety of ion channels, including epithelial channels. For example, it was shown that Rab4 and Rab27 negatively regulate the cystic fibrosis transmembrane conductance regulator CFTR function [39, 40]. Rab5 regulates the trafficking of CFTR from the plasma membrane to early endosomes. From early endosomes, CFTR is recycled back to the plasma membrane through recycling endosomes that are controlled by Rab11 and Rme1 [41]. Rab7 controls the movement of CFTR from early endosomes to late endosomes and also facilitates the trafficking of CFTR to lysosomes. Rab9, in comparison, can drive CFTR from late endosomes to the trans-Golgi network [42]. Van de Graaf and colleagues identified Rab11a as a novel epithelial calcium channel TRPV5- and TRPV6-associated protein [43]. Rab11a colocalizes with TRPV5 and TRPV6 in Ca^{2+}-transporting epithelial cells of the kidney. Here, both Rab11a and TRPV5 are present in vesicular structures underlying the apical plasma membrane [44]. It was observed that these calcium channels interact not only with wild-type small G proteins but also with GDP-locked mutant $Rab11^{S25N}$. Co-expression of a mutant Rab11a protein, locked in the GDP-bound state, resulted in significantly decreased Ca^{2+} uptake caused by diminished channel cell surface expression, indicating a direct role of Rab11a in the trafficking of TRPV5/6 toward the plasma membrane.

Furthermore, Rab proteins control trafficking of chloride channels ClC-2. It was shown that internalization of ClC-2 and endosomal trafficking through the early endosomes was a Rab5 dependent process and that channel retrieval through the recycling endosomes was modulated by Rab11 [45]. Moreover, Rab11 is required to maintain the expression of the water channel aquaporin 2 (AQP2) on the epithelial cell surface [46].

Phosphatidylinositides are one of effectors of the small G proteins. They can be second messengers between small GTPases and ion channels. It was demonstrated that Rab dependent regulation of glucose transporter GLUT4 involved phosphatidylinositol 3-phosphat 5-kinase (PIKfyve), producing $PI(3,5)P_2$ [47]. It was shown that Rab5 was involved in endocytosis of potassium channels KCNQ1 and KCNE1, whereas exocytosis of these channels was mediated by Rab11 [48]. It is intriguing that serum- and glucocorticoid-inducible kinase (SGK1), which plays a significant role in ENaC regulation via phospharylation of Nedd4-2, increases Rab11 dependent traffic of KCNQ1/KCNE1 [13, 48]. In this case there is a contrary mechanism, phosphatidylinositide influences on the small G protein. SGK1 dependent activation of PIKfyve with concomitant increases in $PI(3,5)P_2$ levels modulates Rab11a dependent exocytosis, promoting KCNQ1/KCNE1 insertion into the plasma membrane [48]. Some examples and detailed molecular mechanisms of regulation of ion channels by small Ras, Rho and Rab G proteins are discussed in our recent review [26].

Regulation of ENaC by the Ras Family G Proteins

There are 4 major Ras isoforms of G proteins: Ha-Ras, Ki-RasA, Ki-RasB, and N-Ras. Ras proteins are positioned to initiate a complex arrangement of cellular signaling cascades. Small G proteins in the Ras family are regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. Adaptor proteins, such as GRB2, binding to tyrosine kinase receptor, activate GEF [49]. The model of activation/inactivation of small G proteins and participation of GEFs and GAPs in this regulation is shown in the Figure 2. GEF catalyses conversion of the small GTPase from GDP- to GTP-bound state. Interaction between small G protein and GEF induces release of GDP. Then GTP binds to a free site because GTP concentration in the cell is much more than GDP concentration. Activated small G protein (Figure 2c) interacts with various effectors, including ion channels. After the GTP-bound form of small G protein accomplishes its effects on downstream effector(s), it is converted to the GDP-bound form by the action of GAP. GAP induces hydrolysis of GTP. Thus, this scheme represents a cycle of conversions of inactive (Fig. 2a) and active (Fig. 2c) forms of small G protein.

Ras proteins activate the serine/threonine kinase Raf [50], which then initiates the MAPK cascade, including Raf, MEK and ERK kinases to modulate gene expression [51]. Various signaling molecules, notably growth factors and other agonists of receptor tyrosine kinases, initiate this signaling cascade [52–54]. There is strong

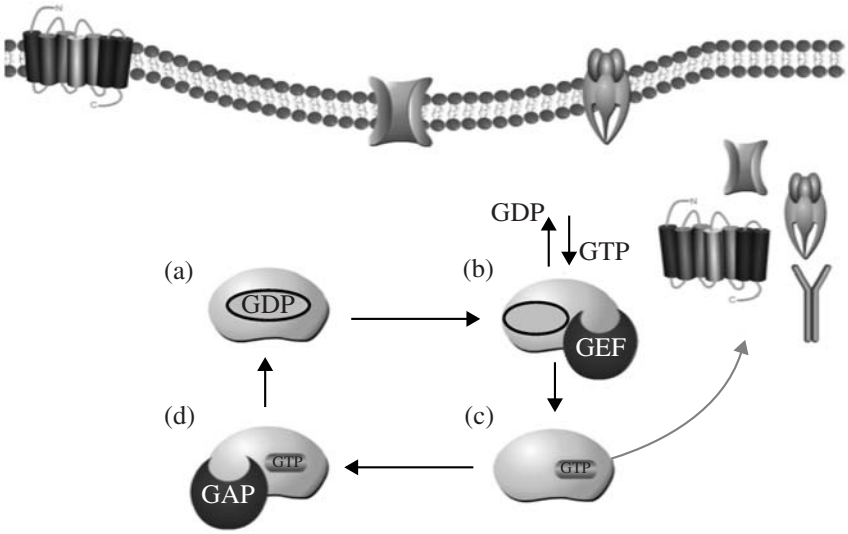

Fig.2. Model of activation/inactivation of small G proteins. Small G proteins act as molecular switches cycling between active GTP-bound and inactive GDP-bound states. Guanine nucleotide exchange factor (GEF) catalyses conversion of the small GTPase from GDP- to GTP-bound state. GTPase-activating protein (GAP) induces hydrolysis of GTP and conversion to the GDP-bound state. (a) Inactive GDP-bound small G protein. (b) Interaction between the small G protein and GEF induces changing GDP to GTP. (c)Active state of the small G protein. (d) Hydrolysis of GTP and conversion of the small G protein to inactive state.

support for Ras proteins involvement in binding and activating Ral GDS, RIN1 and PI3-kinase [55–57].

Some findings show that K-RasA increases the activity of ENaC [58–60]. In renal epithelia of *Xenopus laevis*, aldosterone via control of gene expression increases the level and activity of the small G protein K-Ras [61–63]. Mastroberardino and colleagues [58] have found that expression of K-Ras2A in oocytes of *Xenopus laevis* induced decrease of ENaC number on the cell surface and, simultaneously, increase in channel activity. We demonstrated that expression of K-Ras in Chinese hamster ovary cells (CHO) increases ENaC activity in a GTP-dependent manner [59]. K-Ras increases the activity of channels present in the membrane by increasing channel P_o. K-Ras-dependent increase in ENaC activity is blocked by the PI3-kinase inhibitor wortmannin and mimicked by an overexpression of active PI3-kinase. In contrast, inhibition of other K-Ras effector cascades, including the MAPK by PD98059 and U0126 and Ral/Rac/Rho cascades by Y27632, did not affect K-Ras actions on ENaC. Furthermore, the G12:C40 effector-specific double mutant of Ras, which preferentially activates PI3-kinase, enhanced ENaC activity. In contrast, effector-specific mutants preferentially activating c-Raf kinase (G12:E38) and RalGDS (G12:G37) had no effect. Also we showed that K-Ras increased channel activity independently of effects on ENaC membrane levels. K-Ras most likely directly interacts with ENaC to position PI3-kinase near the channel, although the mechanism of this interaction is unknown [60]. Also, it was shown that $PI(3,4,5)P_3$ directly influenced on P_o of ENaC [64]. In addition it was revealed spatiotemporal coupling between the channel Po and the level of $PI(3,4,5)P_3$ in freshly isolated rat cortical collecting ducts. Inhibition of PI3-kinase with two distinct inhibitors, wortmannin and LY294002, simultaneously decreased the level of $PI(3,4,5)P_3$ on the apical plasma membrane and the activity of ENaC [65]. Together, these observations reveal that K-Ras activates ENaC via PI3-kinase signaling with concomitant increases in $PI(3,4,5)P_3$ levels. Thus, Ras regulation of ENaC can play a role in the control of systemic Na^+ balance.

Regulation of ENaC by the Rho Family G Proteins

Rho GTPases are found in all eukaryotic cells. The most extensively characterized members in the Rho family are Rho, Rac1, and Cdc42. Small G proteins in the Rho family are known to regulate diverse cellular processes, including cytoskeletal organization, cell proliferation, gene transcription, and cell cycling. To date, 30 or so potential effectors of Rho, Rac and Cdc42, including ion channels, have been identified [66]. Additionally, over 60 activators, GEFs, and over 70 inactivators, GAPs, are known for this family [67].

Similar to other small G proteins, Rho GTPases act as molecular switches cycling between active GTP-bound and inactive GDP-bound states (Fig. 2). In the GTP-bound state, Rho GTPases interact with effector molecules, such as Rho kinase, WASP, PI(4)P5-kinase, and PI3-kinase, to initiate downstream responses. Both RhoA and Rac1 as well as Rho-kinase increase $PI(4,5)P_2$ levels by activating PI(4)P5-kinase [68–70].

Rac1 and Cdc42 also interact with PI3-kinase and increase PI(3,4,5)P_3 levels [71–73]. Through these phospholipid second messengers and adapter proteins, Rho small G proteins influence vesicle movement affecting both endocytosis and exocytosis [74–76].

The effects of RhoA on ENaC are similar to those described for K-Ras in the sense that they both increase channel activity. Despite that phospholipids also appear to serve as signaling intermediates in both instances, transduction pathways and mechanism of activation are different [77]. K-Ras increases channel P_o, whereas RhoA increases channel membrane levels. RhoA activates ENaC via Rho-kinase and subsequent activation of PI(4)P5-kinase with concomitant increases in PI(4,5)P_2 levels promoting channel insertion into the plasma membrane [37, 78]. Although insertion of the channel subunits into the plasma membrane is mediated by means of vesicules RhoA did not change cell capacitance [77]. Most likely, plasma membrane area does not change because there is very intensive reverse internalization of inserted vesicles. Recent findings showed that Rac1, another small G protein in the Rho family, also enhanced ENaC activity. It is possible that Rac1, similarly to RhoA, increases ENaC activity via enhancement of the number of the functional channels on the cell surface. However, detailed mechanisms are still unknown. Similar to effect of RhoA on ENaC, Rac1 promotes insertion of TRPC5 into the plasma membrane [79]. This effect on TRPC5 is dependent on Rho-kinase signaling and PI(4)P5-kinase activity, again implicating PI(4,5)P_2 as playing a critical role. However, we can not support that Rac1 and RhoA initiate the same signaling pathway because the effects of small G protein on ion channels are very specific. For example, we demonstrated that Cdc42, belonging to the same family of small G proteins, had no effect on ENaC activity.

Proteins in the Rho family are involved in the regulation of F-actin polymerization. For example, Rho inhibits cAMP-dependent translocation of the water channel aquaporin-2 (AQP2) into the apical membrane of epithelial cells by controlling the organization of the actin cytoskeleton [76, 80]. We demonstrated in the Cos-7 cells that ENaC and actin microfilaments near the membrane did not colocalize. Moreover, treating cells with cytochalasin D or latrunculin B to disrupt microfilaments had no effect on RhoA-mediated insertion of ENaC into the cell surface. In contrast, disrupting microtubules with colchicine led to marked reorganization of ENaC clusters at and near the membrane and significantly slowed channel traffic to the membrane. Despite the fact that ENaC activity depends on actin filaments, we speculate that RhoA promotes ENaC traffic toward the plasma membrane, likely through effects on the microtubules [81].

Thus, Rho signaling and its effects on epithelial ion channels, including control of ENaC and AQP2 membrane levels, may be part of a larger cellular program tightly controlling epithelial transport. RhoA, phosphatidylinositide PI(4,5)P_2 as a downstream effector and cytoskeleton play a key role in the maintenance of the appropriate number of functional channels on the apical plasma membrane.

Regulation of ENaC by the Rab Family G Proteins

Rab proteins, similar to Rho proteins, exist in all eukaryotic cells and constitute the largest branch of the small G protein superfamily. In mammalian cells, more than 60 Rab proteins have been identified. A large body of evidence has been accumulated in support of a role of Rab proteins in endocytosis, exocytosis, and secretions in eukaryotic cells from yeast to human [21]. Rab proteins use the guanine nucleotide-dependent switch mechanism to regulate each of the 4 major steps in intracellular vesicle transport: 1) vesicle budding from the donor membrane, 2) targeting of the vesicle to the acceptor membrane, 3) docking of the vesicle, and 4) fusion of the vesicle with the acceptor membrane. Recently, several groups have demonstrated that Rab5, which is localized in early endosomes, and Rab7, 9, 24, 27, which are localized in endosomes and lysosomes, participated in endocytosis [82, 83]. Rab4, 11, which are presented in recycling endosomes, and Rab3, which is localized in secretory vesicles, were shown to participate in exocytosis [84].

As discussed above, Rab proteins play a key role in the modulation of various epithelial ion channels. Recently we showed that Rab11a is involved in ENaC modulation [85]. Our results clearly demonstrate colocalization Rab11a with ENaC in a plasma membrane region and in vesicles, distributed in cytoplasma. Interestingly, we have observed a colocalization of ENaC with GDP-locked Rab11a^{S25N}. These findings are akin to previously published data about colocalizating TRPV5/6 with Rab11a^{S25N} [43]. We find that Rab11a increases amiloride sensitive current by influencing channel insertion into the plasma membrane. However, mechanism of ENaC regulation by this small G protein is unclear. One of the possible signaling cascades can include SGK1. Seebohm and colleagues [48] have shown that SGK1 promoted Rab11 dependent exocytosis of the potassium channels KCNQ1/KCNE1. Butterworth and colleagues have shown [86] that overexpression of dominant negative mutants of two isoforms of Rab11, Rab11a and Rab11b, resulted in significant loss in both baseline and cAMP-stimulated ENaC activity and decreased the channel number on the cell surface. Knockdown of endogenous Rab11 by RNAi confirmed a role for this small G protein in ENaC regulation. It has been suggested that Rab11b has a greater role as compared to Rab11a in ENaC control, because knockout of Rab11b has a stronger effect on ENaC activity than knockout of Rab11a. However, our findings have shown that the Rab11b effect on the channel is similar to the Rab11a effect. Moreover, we identified a new small G protein from Rab family which has an effect on

amiloride sensitive current. Rab3a significantly increased ENaC activity, but the mechanism of its action is unknown [85].

Saxena and colleagues [87, 88] have demonstrated that Rab 3, Rab 4, and Rab27a inhibited ENaC activity in colonic epithelial HT-29 cells. However, these results are controversial. Rab proteins inhibit ENaC activity via protein-protein interactions. It has also been shown that only the constitutively active, Rab27a^{Q78L}, is successful in regulating ENaC, while the GDP-locked form Rab27a^{T23N} is unable to do so. Rab27a inhibits ENaC function through a complex mechanism involving Munc13–4 and synaptotagmin-like protein SLP-5. Both proteins due to their stronger affinity for Rab27a eliminate negative modulation of Rab27a on ENaC function [89]. In contrast, the GDP-locked form of Rab4 (Rab4^{S22N}) had a greater degree of inhibition of ENaC currents. It is also speculated that the GDP-bound Rab4^{S22N} is involved in the inhibition of exocytosis and, subsequently, decrease of ENaC activity. Rab4^{Q67L} had a dose-dependent effect on ENaC. This constitutively active form of Rab4 leads to an augmentation of amiloride-sensitive currents at low doses and inhibits them at high concentrations. As mentioned above, we showed that Rab3a significantly enhanced ENaC activity and Rab27a slightly decreased it [85]. Moreover, we have tested Rab5 which is presented in early endosomes and which is known to regulate several types of channels. Also we tested Rab38, regulating intracellular traffic, which can also play a role in several pathophysiological processes in kidneys [90, 91]. However, Rab5 and Rab38 had no effects on ENaC activity. In conclusion, despite the huge number of findings regarding Rab proteins effects on ENaC, mechanisms of these effects still require to be investigating.

REGULATION OF ENaC BY PHOSPHATIDYLINOSITIDES

Phosphoinositides serve as important second messengers in a number of intracellular signaling cascades. In many instances phosphatidyloinositides directly bind ion channel targets to modulate channel gating and activity. In 1996 it was shown that cardiac Na^+/Ca^{2+}-exchange and K_{ATP} (Kir6.2) are regulated by phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) [92]. Thus, K_{ATP} activity rapidly decreases when the channels are excised from the cell membrane in an inside-out patch-clamp configuration. This hallmark, termed "run-down", results in part from the loss of $PI(4,5)P_2$ [93, 94]. This decrease demonstrates that $PI(4,5)P_2$ plays a permissive role in the normal function of those channels. Similar dependence on $PI(4,5)P_2$ was described for P/Q-, N- and L- types of calcium channels, GIRK and other ion types channels, including ENaC [95, 96]. Also regulation by phosphoinositides is shown for the TREK-1, HERG, M-type, HCN- and TRP potassium channels [97–101]. Binding and direct activation of the channels and phosphoinositides have an important physiological significance. Disruption of phosphoinositide regulation of ion channels can lead to disease (such as Bartter's, Andersen's and long QT syndromes, congenital hyperinsulinism) [102–106].

The role of phosphoinositide-(PI)-kinases in reabsorpting epithelium layers is being actively investigated. Thus, voltage-clamp assay showed that the non-selective inhibitor of PI3-kinase, vortmannin attenuates sodium transport in the skin of *Rana temporaria* by modulating ENaC activity [107]. PI3-kinase selective blockade in amphibian epithelial cells A6 also leads to the sodium transport attenuation when LY-294002 was applied to apical membrane [108]. There are three ways PI3-kinase influences on ENaC in epithelium. First, numbers of signal cascades which are initiated by the different PI3-kinase products can regulate the channel. Many important regulatory proteins like protein kinase C, small G-proteins and others are a targets for intracellular PI3-kinase and its products [109]. Second, direct interaction between PI3-kinase and ENaC regulates delivering and/or insertion of the channel to apical membrane [110]. Third, there is a direct ENaC regulation by PI3-kinase products of phosphoinositide phosphorylation.

Phosphoinositides and their derivates, phosphatidylinositides are involved in the ion channels regulation by small and heterotrimeric G-proteins. Two small G-proteins, K-Ras and RhoA, activate ENaC functions through those mechanisms. Our and other groups demonstrated that both $PI(4,5)P_2$ and $PI(3,4,5)P_3$ participate in ENaC regulation by small G-proteins. Despite of some ambiguous and even controversial data, strong significance of phosphatidylinositides in ENaC functions is indicated [111–113].

Regulation of ENaC by Phosphatidylinositol-4,5-Bisphosphate

Ma and colleagues provided the first evidence that phosphoinositides directly modulate ENaC activity [111]. This group showed that ENaC in excised, inside-out patches has characteristic run-down. Addition of exogenous $PI(4,5)P_2$ to the intracellular side of ENaC countered run-down. Conversely, scavenging $PI(4,5)P_2$ with an antibody and increasing $PI(4,5)P_2$ 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 $PI(4,5)P_2$ [111]. Similar experiments were performed on CHO cells expressing three ENaC subunits. Figure 3 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 $PI(4,5)P_2$ followed by scavenging with poly-L-lysine [112]. This data confirms a permissive role of $PI(4,5)P_2$ level for ENaC function [64, 111, 112, 114].

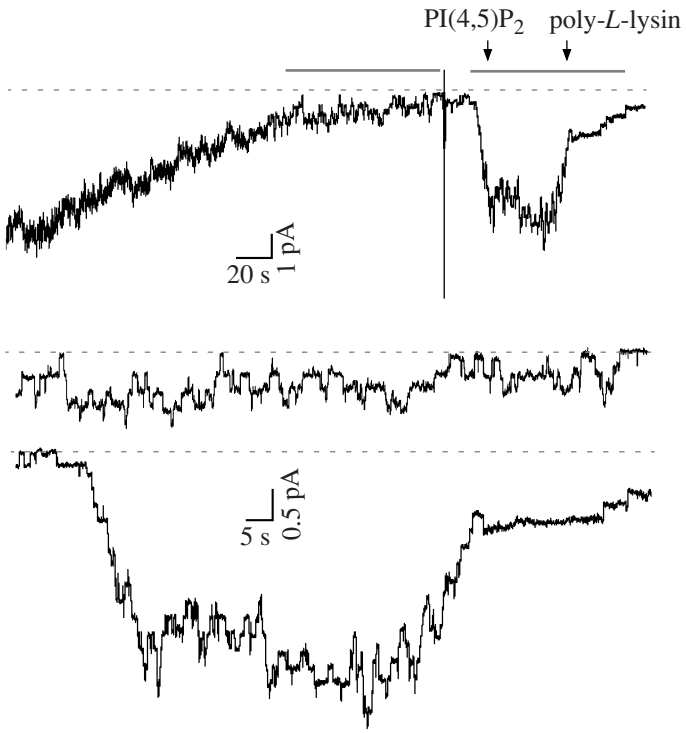

Fig.3. Current trace of ENaC expressed in a CHO cells in an excised, inside-out patch. The patched membrane was clamped to 0 mV. Inward sodium currents are downward with the dashes gray line noting the closed state. Over the course of this experiment, ENaC activity run-down and was then re-activated by addition of 30 μM $PI(4,5)P_2$ to the bath solution (noted with *first arrow*). ENaC activity was consequently decreased upon addition of $PI(4,5)P_2$ 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 $PI(4,5)P_2$ addition at expanded time and amplitude scales (Figure adapted with permission [112]).

Kunzelmann and colleagues [115] showed that stimulating G-protein-coupled purinergic receptors inhibits amiloride-sensitive sodium absorption in airway and immortalized collecting duct epithelial cells (M1 cell line) by promoting $PI(4,5)P_2$ metabolism. Recent studies in $mpkCCD_{c14}$ cell line confirmed that a decrease in apical plasma membrane $PI(4,5)P_2$ levels following purinergic stimulation and consequent alteration of PLC activity is a major regulator of ENaC activity. Moreover, basal ENaC activity is set by resting apical $PI(4,5)P_2$ levels and changes in $PI(4,5)P_2$ levels affect ENaC gating [116]. Therefore, $PI(4,5)P_2$ widely distributed in plasma membrane [117] plays an important role in the ENaC regulation.

Similar to G-protein-coupled receptors, signaling through receptor tyrosine kinases and phosphotyrosine phosphatases is capable of modulating ENaC activity by influencing membrane $PI(4,5)P_2$ levels. Importantly, all studies agree that there is tight spatiotemporal coupling between the levels of $PI(4,5)P_2$ in the membrane and ENaC activity/open probability [118, 119]. Thus ENaC is sensitive to apical plasma membrane $PI(4,5)P_2$ levels which serves as one of mechanisms of rapid channel regulation [116].

Much experimental evidence, particularly the observation that exogenous $PI(4,5)P_2$ prevents decreases in ENaC open probability in excised patches, suggests that $PI(4,5)P_2$ 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. Supporting direct interaction between $PI(4,5)P_2$ and ENaC are co-precipitation studies in which channel subunits segregate with $PI(4,5)P_2$ isolated with anti-$PI(4,5)P_2$ antibody [114, 115]. It was shown, however, that increase of RhoA-dependent ENaC activity was mediated by $PI(4,5)P_2$ influence on a number of channels in the plasma membrane [78]. We suggest that both number of the channels in apical membrane and open probability are involved in ENaC regulation by $PI(4,5)P_2$. It appears that permanent $PI(4,5)P_2$ level alteration, caused by the RhoA mechanism, affects ENaC number in membrane but possible effects on P_o may be hidden during chronic increased $PI(4,5)P_2$ level.

Functional $PI(4,5)P_2$ binding sites have been proposed for several phosphoinositide-sensitive channels,

including Kir and transient receptor potential (TRP) potassium channels, with a high-resolution structure in the absence of $PI(4,5)P_2$ available for these regions in Kir2.1 and 3.1. 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 [120–124]. Rather the β- and possibly γ-subunits of heterotrimeric ENaC play a major role. Support for this position comes from co-precipitation studies in which β- and γ-ENaC subunits are pulled-down by $PI(4,5)P_2$. Also in agreement are the findings of a recent mutagenesis 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 $PI(4,5)P_2$. Authors suggest that the α-subunit is not involved in ENaC regulation by $PI(4,5)P_2$ [114, 115, 118].

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. The regions in the extreme NH_2-termini of β- and γ-ENaC were recently identified as being necessary for $PI(4,5)P_2$ regulation of ENaC [118]. Also there are data suggesting that the regions just proximal to the first transmembrane domains were identified as a binding site for $PI(4,5)P_2$ [115]. This mechanism of ENaC regulation has a direct links with $PI(4,5)P_2$ influence on the other ion channels such as Kir, 2-P, KCNQ and P/Q-, N-type calcium channels [125, 126].

Regulation of ENaC by Phosphatidylinositol-3,4,5-Trisphosphate

A physiological role for PI3-kinase and its product, phosphatidylinositiol 3,4,5-trisphosphate, in modulating ENaC activity is firmly established [65, 127]. This phospholipid kinase is one downstream mediator of aldosterone action on the channel. The steroid hormone aldosterone increases ENaC activity in part by transactivating SGK expression. In response to aldosterone, both the absolute and active levels of SGK increase. Active SGK consequently prevents ENaC internalisation and increases number of channel in the cell apical membrane [128].

Several recent studies demonstrate that, in addition to controlling the levels of ENaC in the plasma membrane, PI3-kinase and $PI(3,4,5)P_3$ signaling also increase the open probability of ENaC. $PI(4,5)P_2$ or $PI(3,4,5)P_3$ injection to *Xenopus laevis* oocytes leads to increasing of the channel gating (P_o) and alteration of transmembrane amiloride-sensitive current density [111]. In native collecting duct principal cells $PI(3,4,5)P_3$ application prevents ENaC run-down in excised, inside-out patches. Moreover, in outside-out patches, a setting where ENaC does not run-down, addition of exogenous $PI(3,4,5)P_3$ increases ENaC activity above basal levels [64, 118, 129]. In CHO cells ENaC open probability is increased by exogenous $PI(3,4,5)P_3$ in an outside-out patch. This parallels findings for which overexpression of active PI3-kinase and inhibition of this kinase increase and decrease ENaC open probability, respectively. In such experiments, changes in ENaC open probability and membrane $PI(3,4,5)P_3$ levels follow identical time-courses indicating close spatiotemporal coupling between the phosphoinositide and the channel [64, 65, 118]. These observations led to the hypothesis that, like $PI(4,5)P_2$, $PI(3,4,5)P_3$ 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 prediction of a physical association between ENaC and $PI(3,4,5)P_3$ initially presented a conceptual challenge. ENaC is more sensitive to $PI(4,5)P_2$ then $PI(3,4,5)P_3$ and $PI(4,5)P_2$ level is 100–1000 times more common in the plasma membrane than $PI(3,4,5)P_3$ [117, 130]. However, we have demonstrated that ENaC had a different binding sites for $PI(4,5)P_2$ and $PI(3,4,5)P_3$ [118, 129]. Using a combination of mutagenesis, electrophysiology and biochemistry, we defined the regions of ENaC important to $PI(3,4,5)P_3$ regulation to the cytosolic portions of the β- and γ-subunits just following the second transmembrane domains, which contain several positive-charged residues. Deletion and charge neutralization of these residues abolished $PI(3,4,5)P_3$ stimulation. In addition, disrupting these tracts prevented co-precipitation of channel subunits with $PI(3,4,5)P_3$. It is interesting that, as for $PI(4,5)P_2$ regulation, α-ENaC subunits again appear to play no role in regulation by $PI(3,4,5)P_3$ [64, 118, 129]. Therefore, we can suggest that there are independent binding sites for $PI(4,5)P_2$ and $PI(3,4,5)P_3$ [131].

CONCLUSIONS

Analysis of literature demonstrates that significant advances in understanding of ENaC regulation mechanisms by small G proteins and phosphatidylinositides have been reached in recent years. ENaC as well as other ion channels are the final target of small G proteins and signaling cascades activated by them. Small G proteins modulate ion channel activity by influencing channel P_o and/or channel trafficking to the plasma membrane. Phosphatidylinositides can be the secondary messengers between small GTPases and final effectors. Figure 4 shows possible model of ENaC regulation by small G proteins K-Ras, RhoA and Rab11. K-Ras via PI3-kinase and $PI(3,4,5)P_3$ modulates P_o. RhoA and Rab11 play a significant role in channel traffic towards the plasma membrane. It has been shown that several other small G proteins are involved in con-

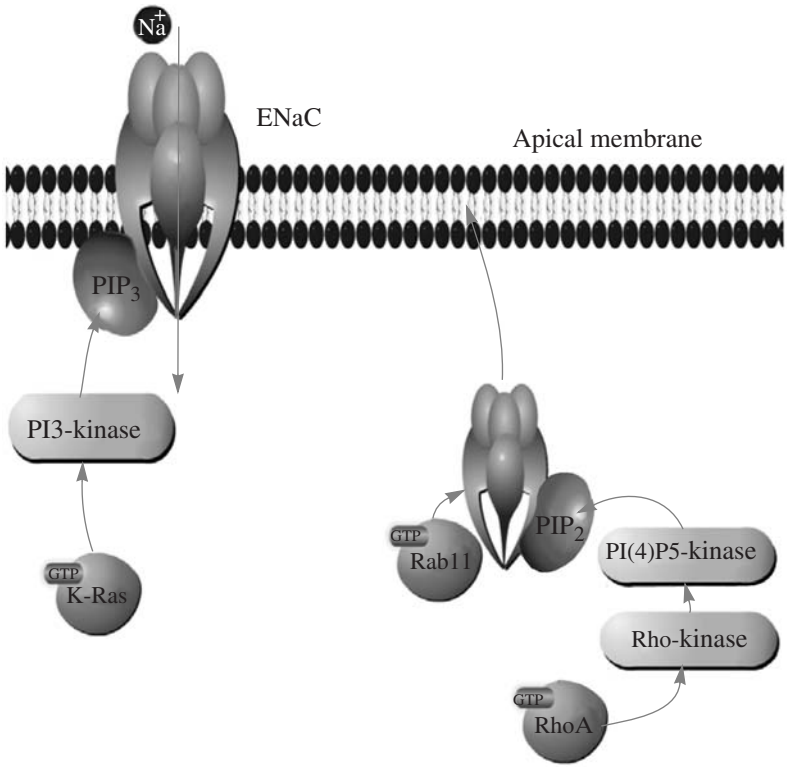

Fig.4. Relative model illustrating possible mechanisms of ENaC regulation by small G proteins.

trol of ENaC activity and their effect is specific. Thus, ENaC regulation by small G proteins and phosphatidylinositides appears to be widespread and plays an important role in maintenance of ENaC-mediated Na^+ homeostasis. However, mechanisms of ENaC modulation and their associated downstream signaling cascades require further investigation.

ACKNOWLEDGMENTS

The article was supported by AHA (SDG 0730111N) and ASN (Carl W. Gottschalk Research Scholar Grant).

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