Published in final edited form as:
Exp Cell Res. 2012 May 15; 318(9): 1011–1019. doi:10.1016/j.yexcr.2012.02.024.

Epithelial Na^+ Channel Regulation by Cytoplasmic and Extracellular Factors

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Abstract

Electrogenic Na^+ transport across high resistance epithelial is mediated by the epithelial Na^+ channel (ENaC). Our understanding of the mechanisms of ENaC regulation has continued to evolve over the two decades following the cloning of ENaC subunits. This review highlights many of the cellular and extracellular factors that regulate channel trafficking or gating.

Keywords

epithelial Na^+ channel; fluid volume; regulation; protein kinases; phosphatidylinositol

The aldosterone-sensitive distal nephron represents the final site within the nephron where filtered Na^+ is reabsorbed. ENaCs are expressed in principal cells in the late distal convoluted tubule, connecting tubule and through the collecting duct, and are the major pathway for Na^+ entry across the apical plasma membrane. The regulated reabsorption of Na^+ via ENaC in the distal nephron has a key role in the control of extracellular fluid volume, blood pressure, and renal K^+ secretion. ENaCs are also expressed within the airways [1-5], where they have a key role in the modulating airway fluid volume [6, 7], an important factor facilitating mucociliary clearance [7, 8]. ENaCs are also expressed in the distal colon, sweat ducts, salivary ducts, inner ear, lingual epithelium, keritinocytes, lymphocytes and vascular smooth muscle. ENaC expression has also been reported in endothelium and in various sites within the eye (epithelia within retina, lens, and pigmented ciliary body and iris [9-17]. The functional role(s) of ENaCs within many of these tissues is, at present, unclear.

The role of in ENaC in the control of blood pressure is perhaps best exemplified by two rare inherited disorders. Liddle's syndrome, an autosomal dominant disorder characterized by extracellular fluid volume expansion, hypertension and hypokalemia, which is associated with gain of function mutations within the channel's β or γ subunit [18-26]. Pseudohypoaldosteronism type I, an autosomal recessive disorder characterized by volume depletion, hypotension, and hyperkalemia is associated with loss of function ENaC mutations [27]. Some common ENaC polymorphisms are associated with altered channel activity [28-31], and may segregate with blood pressure in selected populations (e.g., βT594M) [32]. Disorders of mineralocorticoid and glucocorticoid metabolism, as well as receptor mutations, are associated with increases in ENaC activity and hypertension [33-35]. In addition, cystic fibrosis is characterized by a decrease in airway fluid volume and increased ENaC activity in airway epithelia. Channel activation reflects, in part, enhanced

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proteolysis of channel subunits [36-38]. The increase in ENaC activity in cystic fibrosis may contribute to a reduced airway surface liquid volume with associated increases in the viscosity of airway fluids and reduction in mucociliary clearance [39-42].

ENaC subunit structure

ENaC subunits are members of the ENaC/Degenerin gene family [43-45]. ENaCs from mammalian kidney tissues are comprised of three subunits, α, β, and γ that likely form a heterotrimer [46], analogous to the structure of a related gene family member, acid sensing ion channel 1 (ASIC1) [47].

The three subunits share modest (~30% to 40%) sequence identity. Each subunit has two membrane spanning helices (TM1 and TM2) resulting in cytoplasmic amino and carboxyl termini [48, 49]. The cytoplasmic domains have sites that are phosphorylated by specific kinases, have specific motifs that direct protein-protein and protein-lipid interactions that affect channel gating or trafficking, and have sites that may directly influence channel gating or trafficking (Figure 1). Between the TM helices in the linear sequence lies a large extracellular region. Based on homology to ASIC1, this region is organized in distinct domains with well-conserved β-sheet domains at the center of the fold and poorly conserved α-helical domains surrounding the protein core (Figure 1) [50, 51]. This region is responsible for extracellular ligand-dependent gating, proteolytic activation, and mechanosensitivity.

ENaC regulation by cytoplasmic factors

The cytoplasmic region of ENaC is comprised of the amino and carboxyl termini of each subunit. These cytoplasmic domains serve as sites of chemical modification, protein binding, and interactions with components of the plasma membrane, often in response to cell signals that regulate ENaC activity by either changing the number of channels at the cell surface or by changing the open probability of the channel. In addition, the carboxyl termini serve to link the channel to the cytoskeleton by binding α spectrin and possibly actin [52, 53]. Finally, sites within this region have been implicated in the functioning of the channel, having roles in ion conduction, ion selectivity, or gating.

Ubiquitination

Mutations in individuals with Liddle's syndrome suggested that a proline-rich region (Figure 1B) within the carboxyl-termini of the β or γ subunit have an important role in the regulation of channel trafficking [18-21, 54, 55]. Staub, Rotin and colleagues used this region of the β subunit of ENaC as bait in a yeast two-hybrid screen to identify proteins that interact with ENaC and isolated an E3 ubiquitin ligase, Nedd4 whose WW domains serve to mediate the interaction between ligase and ENaC [22, 56, 57].

Nedd4-2, the isoform that has a key role in modulating channel activity, is expressed in tissues that also express ENaC. The Nedd4-2 WW domains interact in vitro with a proline-rich region that contains a proline-tyrosine (PY) motif in the carboxyl terminus of ENaC subunits [58-60]. Co-expression studies with Nedd4 and ENaC in oocytes demonstrated that Nedd4 decreases ENaC surface expression and that this is dependent both on the E3 ligase domain of Nedd4 and on the presence of lysine residues on the amino-termini of channel subunits that are the sites of ubiquitin modification [56]. Nedd4-2 dependent ubiquitination of channel subunits occurs at the cell surface, targeting channels for internalization [61-63]. Following internalization, channels recycle to the plasma membrane or are degraded. Deubiquitination appears to be an important step that facilitates the recycling of channels to

the plasma membrane. Recently, ENaC was identified as a substrate for two deubiquitinating enzymes, UCH-L3 and Usp2-45 [64-66].

The interaction of Nedd4-2 with its target proteins is modified by kinases that phosphorylate the ubiquitin ligase or ENaC at defined sites. For example, phosphorylation of Nedd4-2 by serum and glucocorticoid-regulated kinase (sgk1) or by protein kinase A (PKA) results in the recruitment of an adaptor 14-3-3 protein that disrupts the binding of Nedd4-2 with the channel and subsequent ubiquitination and channel internalization [67-73]. Sgk1 also increases channel open probability by directly phosphorylating the carboxyl-terminus of the α subunit [74]. PKA also activates ENaC by increasing the delivery of channels from an intracellular pool to the plasma membrane and by increasing channel open probability [75, 76]. The PKA-dependent delivery of channels to the plasma membrane may involve dephosphorylation of sites within the channel that are targeted by ERK [77]. Other kinases, such as IκB kinase-β [78], phosphorylate Nedd4-2 and activate ENaC.

In contrast, there are several kinases that enhance the interaction of Nedd4-2 with the channel and inhibit channel activity. For example, the cellular energy sensor AMP-activated protein kinase (AMPK) [79, 80] inhibits ENaC in a Nedd4-2 dependent manner. While Nedd4-2 is an AMPK target, the mechanism by which AMPK enhances the inhibitory efficacy of Nedd4-2 has not been elucidated. ERK directly phosphorylates βT613 and γT623 in the immediate vicinity of the PY motif essential to Nedd4-2 binding [81]. ERK-dependent phosphorylation of ENaC facilitates interactions between the channel and Nedd4, thereby inhibiting ENaC activity. JNK1 was recently shown to phosphorylate Nedd4-2 and appears to modulate its activity [80].

The glucocorticoid-induced leucine zipper protein GILZ1 is an aldosterone-induced protein that activates ENaC by inhibiting MAP kinase signaling and ERK activation. Soundararajan, Pearce and colleagues have shown that sgk1, GILZ1, Nedd4-2, as well as members of the Raf-MAP kinase signaling pathway are present within a complex that is associated with ENaC, termed the ENaC regulatory complex. GILZ appears to have an important role in recruiting sgk1 to this complex and preventing rapid degradation [62, 82-84].

G protein-coupled receptor kinase, Grk2, has the opposite effect when it phosphorylates S633 in carboxyl-terminus of the β subunit [85]. Phosphorylation at this site renders the channel insensitive to regulation by Nedd4-2, resulting in increased surface expression and channel activity and explains why increased Grk2 activity has been associated with hypertension [86]. Grk2 also activates channel activity in a manner that is dependent on Gαq/11 but independent of kinase activity [87].

Other kinases

Although many kinases regulate ENaC, evidence for direct phosphorylation of ENaC subunits exists for a small subset of these. Protein kinase C (PKC) activation is associated with an increase in phosphorylation of the cytoplasmic carboxyl-termini of the β and γ subunits in both live cells and isolated membranes [88, 89], lowering both channel open probability and the expression of β and γ subunits [89, 90]. PKC activity is required for insulin regulation of ENaC [91]. At present, it is unclear whether PKC isoforms directly phosphorylate channel subunits. The PKC δ isoform seems to differentially regulate the αA/T663 human ENaC polymorphism, although T663 is not a PKC δ phosphorylation site [92].

Casein kinase 2 activates ENaC in association with phosphorylation of intracellular C-terminal sites βS631 and γT599 [93, 94]. Activation likely results from reduced Nedd4-2 binding, increasing the number of channels at the cell surface. Casein kinase 1 also

differentially regulates the αA/T663 human ENaC variant, although the effect is indirect [95].

Members of the WNK (with no lysine (K)) kinase family modulate ENaC activity. The N termini of all four WNK kinases activate ENaC. This appears to reflect, in part, activation of sgk1 [96]. Furthermore, sgk1 phosphorylates and appears to modulate WNK4 activity [97]. While one group suggested that full length WNK1 and WNK4 have been reported to activate ENaC [96], another group showed that WNK4 inhibits ENaC [97, 98].

Lipids

The anionic, cellular phosphoinositides phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$) and phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) affect ENaC function. Patch clamp studies have shown that $PI(4,5)P_2$ and $PI(3,4,5)P_3$ can directly activate channels through binding to tracks of cytoplasmic basic residues in the β and γ subunits [99-103]. Increased levels of these phosphoinositides are associated with increased ENaC open probability. $PI(4,5)P_2$ was also associated with increases in ENaC surface expression [104]. In addition to its well defined effects on ENaC gating, the local generation of $PI(4,5)P_2$ via phosphatidylinositol 4-phosphate 5-kinase α enhances ENaC endocytosis [105].

$PI(4,5)P_2$ regulation of ENaC appears to be a convergence point for various regulatory pathways. The activation of luminal purinergic receptors, results in activation of phospholipase C, hydrolysis of $PI(4,5)P_2$ and an inhibition of ENaC activity [106, 107]. Likewise, epidermal growth factor inhibits ENaC by activating receptor tyrosine kinases, which leads to depleted $PI(4,5)P_2$ levels [108].

Methylation reactions have been implicated in the activation of ENaC by aldosterone [109, 110]. Aldosterone stimulates carboxylmethylation of proteins and phospholipids, and inhibition of these reactions blunts the ENaC response to steroid stimulation [111]. Two potential target proteins of methyltransferases are k-ras and the ENaC β subunit. Induction and processing of k-ras appears to be important for regulation of ENaC in A6 cells [112]. Methylation of ENaC in planar lipid bilayers has been shown to lead to an increase in open probability of the channel [113]. Edinger and co-workers have identified a methyltransferase that targets the β subunit of ENaC [114].

In addition, palmitoylation of ENaC β and γ subunits at specific cytoplasmic cysteine residues was recently reported [115]. β subunit palmitoylation increases channel open probability, but does not affect the number of channels at the membrane. Only a small percentage of channels were reported to be palmitoylated, suggesting that ENaC palmitoylation may occur at specific locations within cells. The palmitoyltransferase(s) that regulate the modification of channels by palmitate, as well as the upstream regulators of this process have not been reported.

Sodium

Increases in intracellular Na^+ reduce both ENaC surface expression as well as open probability, a process referred to as feedback inhibition. The reduction in surface expression is dependent, in part, on G proteins and on Nedd4-2 and related ubiquitin ligases [116-118]. The reduction in channel open probability may be due to a reduction in proteolytic processing of ENaC subunits (see below) [119, 120].

Trafficking itinerary

The assembly and export of channels from the ER, forward trafficking of assembled channels through the biosynthetic pathway to the plasma membrane, retrieval from the plasma, and the recycling or degradation of internalized channels are events that require specific accessory proteins. Changes in channel trafficking at any of these steps may alter the cellular or surface pool of channels. A growing number of proteins that influence ENaC trafficking have been identified, including ER chaperones [121, 122], members of the rab family of GTPases [123-126], SNARE proteins [127-130], ubiquitin ligases and deubiquitinating enzymes (see above), and members of the ESCRT (endosomal sorting complexes required for transport) complex that targets internalized channels for degradation [131].

ENaC regulation by extracellular factors

The extracellular region of proteins in the ENaC/Degenerin family confers sensitivity to exogenous factors. Based on homology to ASIC1, this region of ENaC is likely organized into several discrete domains (see Figure 1) [47, 51]. At the center of the fold are two β-sheet domains termed the “palm” and “β-ball”. These domains are well conserved across the gene family and form the core of the trimer. Stemming from this foundation are the “finger”, “thumb”, and “knuckle” domains. These are likely formed by helical and coiled regions and are poorly conserved among members of the family. The thumb domain is also characterized by ten conserved cysteines that form five disulfide bonds in the ASIC1 structure, confirming a disulfide bridged helical ladder in the α subunit of ENaC that had been proposed based on functional data [132, 133]. Several extracellular or external factors influence ENaC activity at the cell surface: ions, proteolytic cleavage, and mechanical stress [51].

Sodium, chloride and protons

Na^+ and Cl^- both have physiologic roles in ENaC regulation, while the effect of H^+ is species-specific. Extracellular Na^+ has long been known to acutely down-regulate ENaC activity, a process referred to as Na^+ self-inhibition [134]. This is an allosteric inhibitory effect that results in a reduction in channel open probability [135, 136], and is distinct from the slower “feedback inhibition” due to increases in the intracellular Na^+ concentration [116-118]. The extracellular Na^+ binding site(s) and the mechanism of transduction remain undefined. Cl^- has recently been reported to modulate ENaC activity [137]. The ASIC1 structure revealed 3 Cl^- binding sites at each of the intersubunit interfaces [47]. Testing analogous ENaC residues at these sites defined by the interface of the thumb and palm domains of different subunits suggests that two of these are allosteric effector sites for Cl^- [138]. Protons activate human, but not rodent ENaCs [139]. In additional, divalent cations such as Zn^{2+} and Ni^{2+} modulate ENaC activity [140, 141].

Proteases

Na^+ channel subunits undergo assembly in the endoplasmic reticulum, where core, high mannose asparagine-linked glycans are added at specific sites and are later modified to complex-type endoglycosidase H-resistant forms [142, 143]. ENaC α and γ subunits are also cleaved by the pro-protein convertase furin in the biosynthetic pathway [143, 144]. The α subunit is cleaved twice by furin releasing an imbedded inhibitory tract, which partially activates the channel by increasing its open probability [135, 145-147]. The γ subunit is cleaved once by furin. Cleavage of the γ subunit by a second protease at a site distal to the furin cleavage site releases a second, distinct inhibitory tract that further activates the channel [146, 148-150]. Proteases that cleave the γ subunit at a distal site and activate the

channel have been found on the plasma membrane and within the epithelial lumen (for reviews, see [149, 151]).

Shear stress

ENaCs are expressed at the apical surface of cells in the distal nephron, where they are subjected to variable mechanical stresses due to variable fluid flow rates. ENaC, like several members of its gene family, is mechanosensitive and is activated by laminar shear stress [152-155]. The structures involved in the mechanical stress response are not yet defined, but altering membrane composition had little effect, making membrane deformation an unlikely mechanism [156]. Conversely, structures that have been implicated in the response of the channel to other ligands appear to modulate the stress response [157-159].

Summary

Mechanisms of ENaC regulation are complex, involving a myriad of both intracellular and extracellular factors. It is notable that the vast majority of these regulatory mechanisms, many of which are in the pathways that we now know are modulated by ENaC regulatory hormones such as aldosterone and vasopressin, have been described over the past two decades. There are clear gaps in our understanding of many of these regulatory mechanisms. For example, how do PKC isoforms modulate ENaC activity? How are the multiple phosphorylation sites within Nedd4-2 coordinated to alter its regulatory functions? How do external and internal factors influence structural transitions that affect channel gating? It is likely that these and other questions will be answered over the next decade.

Acknowledgments

This work was supported by National Institutes of Health grants DK051391, DK065161 DK078734 and DK079307 (the Pittsburgh Center for Kidney Research).

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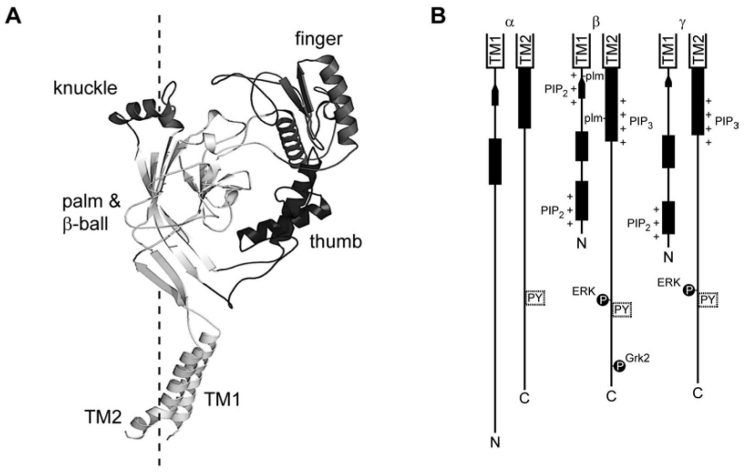

Figure 1.

(A) Model of the extracellular and transmembrane regions of the α subunit of ENaC [50]. Domains within the extracellular region are noted. The dashed line indicates the pseudo three-fold symmetry axis. (B) Linear depiction of the cytoplasmic regions of the three mouse ENaC subunits. Secondary structure prediction was performed using Jpred3 [160]. Predicted α helices (rectangles) and β-strands (arrows) are noted. PY motifs, ERK and Grk2 phosphorylation sites, palmitoylation (plm) sites, and PI(4,5)P_2 and PI(3,4,5)P_3 binding sites are indicated.