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ENaC regulation by proteases and shear stress

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Abstract

Epithelial Na⁺ channels (ENaCs) are comprised of subunits that have large extracellular regions linked to membrane spanning domains where the channel pore and gate reside. A variety of external factors modify channel activity by interacting at sites within extracellular regions that lead to conformational changes that are transmitted to the channel gate and alter channel open probability. Our review addresses two external factors that have important roles in regulating channel activity, proteases and laminar shear stress.

Keywords

Furin; prostaticin; plasmin; protease inhibitors; mechanosensation; degenerins

EPITHELIA SENSE FACTORS IN THE EXTERNAL ENVIRONMENT

Epithelial layers cover surfaces and line the cavities of major organs (e.g., skin, airway and alveoli, gastrointestinal tract, kidney tubules, and pancreatic ducts). Epithelial cells are packed in sheets that are held together via tight junctions, creating a barrier with selective permeability separating the internal environment from the external environment. In addition to functioning as a barrier, epithelia serve other essential roles such as absorption or secretion of solutes.

Epithelia are also exposed to a variety of external factors that may affect specific cellular properties. For example, proteases in the external environment may selectively modify epithelial proteins. Epithelia are potentially exposed to an array of mechanical forces, including cell-associated forces such as osmotic pressure, membrane stretch, and the forces generated by cytoskeletal movements, as well as external forces such as hydrostatic pressure, shear stress, twisting, compression and high-frequency vibrations [1-3]. Within the nephron, tubular epithelial cells are constantly exposed to forces generated by luminal flow, including shear stress and circumferential stretch. Our review addresses the roles of proteases and shear stress in regulating the activity of the epithelial Na⁺ channel (ENaC) that is found in the apical membrane of high-resistance, Na⁺-transporting epithelia.

EPITHELIAL Na⁺ CHANNELS

ENaCs mediate Na⁺ uptake across apical or luminal membranes, the rate-limiting step of transepithelial Na⁺ transport. They are found in many salt-reabsorbing epithelia, including the distal nephron, airway and alveoli, urinary bladder, and distal colon. ENaC-mediated Na⁺ absorption in the distal nephron has an essential role in extracellular volume

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homeostasis and blood pressure regulation, while Na⁺ absorption in the airway has a key role in regulating airway surface liquid volume and mucociliary clearance [4, 5].

ENaCs are members of the ENaC/degenerin family of ion channels. They are highly Na⁺ selective channels that are inhibited by the diuretic amiloride and are comprised of three structurally related subunits, referred to as α , β and γ [4, 5], which share a similar secondary structure consisting of a large extracellular region linked to two membrane spanning domains (TM1 and TM2), and short intracellular N- and C-termini. The resolved structure of a related family member, an acid sensing ion channel (ASIC1), has provided important insights into the structural organization of the extracellular regions of members of this ion channel family [5, 6]. The extracellular region of ASIC1 has a highly ordered structure containing domains formed either by β strands or α helices. The structure resembles an outstretched hand containing a ball. Domains within the structure are aptly named wrist, finger, thumb, palm, knuckle and β -ball [6]. Jasti et al. proposed that proton-dependent gating of ASIC1 is initiated by binding to a proton-sensor located in the interface between the thumb and finger domains. This triggers a series of conformational changes that are transmitted to the transmembrane domains, where the pore and gate likely reside [6-8]. Our group recently published a comparative model of the α subunit of ENaC (Fig 1), which has provided important insights regarding the mechanism by which proteases activate ENaC (discussed below [5, 9, 10]). It is likely that external factors such as proteases and laminar shear stress (LSS) drive changes in the conformation of the extracellular regions of ENaC subunits that are transmitted to the channel gate.

ENaCs ARE PROTEASE-REGULATED CHANNELS

Initial reports 30 years ago demonstrating that selected protease inhibitors inhibit electrogenic epithelial Na⁺ transport provided the first clue that proteases have an important role in modulating ENaC activity [11]. A series of studies from Vallet, Chraïbi, Horisberger, Rossier and colleagues provided the first evidence that proteases, such as trypsin and chymotrypsin, activated ENaCs expressed in oocytes as well as in epithelia [12, 13]. Vallet, Vuagniaux and co-workers identified a group of serine proteases that activated ENaC that were termed channel activating proteases, or CAPs [13-15]. Subsequent studies from many groups, including ours, have addressed a number of important questions regarding mechanisms by which proteases activate ENaC. These studies have been summarized in several recent reviews [5, 16-18].

Proteases activate ENaC by cleaving the α or γ subunits at defined sites within their finger domains that flank imbedded inhibitory tracts [19-21]. As the release of an inhibitory tract activates the channel by increasing its open probability (P_o), subunits must be cleaved at least twice at sites flanking the inhibitory tract to be activated [17]. Release of the γ subunit inhibitory tract has a more robust effect on channel activation than release of the α subunit inhibitory tract [22]. Many proteases have been implicated in cleaving and activating the channel. Furin, a key protease in regards to ENaC activation, is a member of the proprotein convertase family of serine proteases that resides primarily in the trans-Golgi network and processes proteins transiting through the biosynthetic pathway [23]. Furin cleaves the α subunit twice, releasing a 26 residue fragment [19, 20]. Mouse ENaCs expressed in CHO cells that lack furin are inactive and lack α and γ subunit processing [19]. Mutation of the α subunit furin consensus sites dramatically reduces channel activity as well as α subunit proteolytic processing [19]. Furthermore, mutating the α subunit by simply deleting the 26 residue tract between the furin sites is sufficient to activate channels, even if the proximal furin site has also been mutated so that the α subunit is not cleaved [20].

As the γ subunit is cleaved by furin only once, cleavage by a second protease at a site distal to the furin site is required to release the inhibitory tract and activate ENaC [19, 21]. An increasing number of proteases, including prostasin (also referred to as CAP1), transmembrane protease serine 4 (also referred to as TMPRSS4 or CAP2), matriptase (or CAP3), elastase, kallikrein and plasmin have been shown to activate ENaC by inducing cleavage of the γ subunit at sites distal to the furin site [22, 24-30]. For example, prostasin induces cleavage of the mouse γ subunit at a polybasic RKRK tract and in conjunction with furin cleavage releases a 43 residue tract [21]. Mutating the γ subunit by deleting the 43 residue tract between the furin and prostasin sites generates channels with a very high P_o , even if the proximal furin site is also mutated so that the γ subunit is not cleaved [21].

As protease activation is associated with the release of imbedded inhibitory tracts, it was not surprising to find that synthetic peptides corresponding to these 26 (α subunit) and 43 (γ subunit) residue tracts inhibit the channel. Carattino, Passero and colleagues identified limited regions within these peptides that retained inhibitory properties, including an 8 residue (LPHPLQRL) peptide derived from the mouse α subunit and an 11 residue (RFLNLIPLLVF) peptide derived from the mouse γ subunit [22, 31]. Work from our group, led by Kashlan, has provided new insights regarding the mechanisms by which these imbedded tracts inhibit the channel [5]. This required generation of a structural model of the extracellular region of the α subunit. We had noted that the imbedded α subunit inhibitory tract and its corresponding synthetic 8-mer peptide interact at overlapping sites within the channel [9]. This observation provided us with an approach to develop a homology model of the α ENaC, based in large part on the resolved ASIC1 structure [32]. The α ENaC has modest sequence identity to ASIC1 throughout most of the extracellular region. However, identity is particularly poor in the finger domain where the α subunit has 73 additional residues. We examined the response of channels with Trp substitutions in the finger and thumb domains of the α subunit to the 8-mer LPHPLQRL inhibitory peptide [32]. This information was used to define distance constraints within the finger and thumb domains. A structural model of the α subunit extracellular region was generated based on the ASIC1 structure and these distance constraints [32]. The model placed the inhibitory tract at a finger-thumb domain interface (Figure 2). We successfully cross-linked channels with Cys substitutions at key sites in the α subunit to Cys derivatives of the 8-mer peptide with bifunctional Cys-reactive reagents, confirming key elements of our model [9, 10]. Furthermore, we were able to confine the channel to a low activity state by cross-linking sites at the thumb-finger interface [10]. Our data suggest that ENaC gating is influenced by dynamic changes at the finger-thumb interface. Constraining movement at a finger-thumb interface by an intrinsic inhibitory tract or an inhibitory peptide favors a closed state [33]. Proteases release this constraint by liberating inhibitory tracts [9, 10].

As discussed above, furin cleaves the γ subunit once, which allows a second protease to cleave the γ subunit and release its inhibitory tract [19, 21]. Defining the key proteases that cleave the γ subunit and activate ENaC under specific pathophysiological states, as well as the key protease inhibitors that modulate their activity has been challenging. Prostasin was suggested to have a role in γ subunit processing in the setting of volume depletion and/or aldosterone administration [34, 35]. It has also been proposed to have a role in ENaC activation in alveolar cells and in alveolar fluid clearance [36]. Plasmin was suggested to have a role in γ subunit processing in the setting of nephrotic syndrome [29, 31, 37, 38]. Elastase was suggested to have a role in γ subunit processing in the airways of individuals with cystic fibrosis and airway inflammation [39, 40].

ENaCs ARE REGULATED BY MECHANICAL FORCES

ENaCs are members of a family of ion channels that includes mechanosensitive ion channels identified in *Caenorhabditis elegans* (*C. elegans*) [41]. Mutations within these *C. elegans* channels have led to both touch insensitivity as well as degeneration of mechanosensory neurons [42]. ENaCs and related family members are expressed at sites within tissues that sense mechanical stimuli, such as foot pad nerve endings, arterial baroreceptors, and vascular endothelial cells [43-47].

Early studies of ENaC mechanosensitivity focused on the response of reconstituted ENaCs to hydrostatic pressure or membrane stretch. For example, when the α subunit of bovine ENaC was incorporated into planar lipid bilayers, application of hydrostatic pressure led to a marked increase in channel activity [48]. Furthermore, a transient increase of inward currents was observed in *Xenopus* oocytes expressing the bovine α subunit following exposure to membrane stretch induced by either hypotonic fluids or by fluid microinjection [48]. In agreement with these observations, Kizer et al. noted an increase in channel activity in fibroblasts expressing the α subunit of rat ENaC when negative pressure was applied to a cell-attached patch [49]. Subsequent work from Benos' group confirmed that single channel Po of rat $\alpha\beta\gamma$ channels reconstituted in lipid bilayers increased in response to hydrostatic pressure [50]. Awayda and Subramanyam reported that rat ENaCs expressed in oocytes were not sensitive to osmotically induced cell swelling or fluid microinjection [51], but were inhibited by reducing cell volume. In contrast, Ji and colleagues reported that amiloride-sensitive Na^+ currents were altered by changing the osmolality of the solution bathing the oocyte, with currents increasing in response to reducing cell volume and falling in response to cell swelling [52].

Increases in ENaC activity in response to increases in hydrostatic pressure have been observed in native epithelia. Palmer and Frindt examined the effect of mechanical forces on ENaC gating in rat cortical collecting ducts (CCDs) by applying suction to a pipette while recording in a cell-attached patch mode [53]. Mechanical perturbation via negative pressure up to 80 mmHg elicited an increase in channel Po in six out of 21 patches [53]. The authors speculated that variability in the response could reflect differences in the mechanically induced membrane deformations.

Epithelia lining the urinary bladder are subject to changes in hydrostatic pressure and stretch during bladder filling and emptying. Uroepithelia express ENaCs and provide a system to examine how channels respond to hydrostatic pressure. Rapid increases in hydrostatic pressure across the mucosal surface of rabbit bladder uroepithelium were associated with increases in ion conductance that were, in part, amiloride sensitive and likely mediated by ENaC [54-56]. Repeated increases of hydrostatic pressure induced increases in short circuit current and conductance, an effect that was blocked by mucosal amiloride suggesting that increases in hydrostatic pressure enhance ENaC activity [56].

ENaC REGULATION BY SHEAR STRESS

Cells lining tubular lumen are often subjected to shear stress. Ion channels have an important role in sensing mechanical stimuli such as shear stress and activating cellular signaling pathways that in turn alter cellular properties [57]. In this context, perhaps the best studied examples are endothelial cells that line blood vessels and epithelial cells that line the urinary tract and airway. Variations of flow rates within different segments of the nephron regulate the transepithelial transport of Cl^- , K^+ , and Na^+ [58-65], including ENaC-mediated Na^+ transport. It has been suggested that the flow-dependent activation of both ENaC and large conductance, Ca^{2+} activated K^+ channels provides a mechanism to enhance renal K^+

secretion and may reflect an adaptive response in animals to an intermittent consumption of a protein- and K^+ -rich meal [66].

In 2001, our group in collaboration with Satlin's group showed that increases in rates of tubular perfusion of freshly isolated rabbit CCDs were associated with increases in the rate of net Na^+ absorption. Amiloride-sensitive Na^+ currents in oocytes expressing mouse ENaCs were also stimulated by bath perfusion [65]. These findings provided evidence that ENaC is activated by flow mediated mechanical stress and have been confirmed in different expression systems. For example, amiloride-sensitive currents were stimulated by flow in CHO cells expressing mouse ENaCs [67]. To address the question of the type of force responsible for these observations, Carattino and coworkers examined ENaC's response to laminar shear stress (LSS) in oocytes expressing mouse ENaCs. LSS was applied to oocytes via a vertical perfusion through a submerged jet. Whole cell Na^+ currents increased in response to the initiation of vertical flow, suggesting that ENaCs are activated by LSS [68]. Channel activation in response to LSS is a common response of ENaCs from a variety of different species, including mouse, human, rat and *Xenopus* [69-71]. Together, these studies support the notion that shear stress activates ENaCs in native tissues and exogenous expression systems.

In addition to exerting a direct effect on the channel, LSS affects other cellular processes that may influence ENaC as well as other channels in the distal nephron. For example, LSS increases intracellular Ca^{2+} , which has a role in activating large conductance Ca^{2+} -activated K^+ channels [72]. LSS-induced increases in intracellular Ca^{2+} do not appear to have a role in modulating ENaC activity [73]. LSS also enhances the release of cellular factors into the lumen, such as ATP [74-76]. ATP release dampens ENaC activity, presumably through activation of apical P2Y2 receptors and reducing levels of phosphatidylinositol 4,5-bisphosphate [74, 77, 78].

SHEAR STRESS ACTIVATES ENaC BY INCREASING CHANNEL OPEN PROBABILITY

A number of lines of evidence suggest that LSS-mediated channel activation is a result of an increase in channel P_o . The rapid time course for changes in whole cell Na^+ currents following the initiation and termination of LSS were most consistent with changes in channel P_o . The effects of LSS on ENaC mutants with altered gating properties were also consistent with changes in P_o . For example, LSS failed to increase the whole cell Na^+ currents of mutant channels with a high intrinsic P_o [68]. Mutations in the pore region altered the channel's response to LSS [79]. Human channels composed of $\delta\beta\gamma$ subunits have a high intrinsic P_o [80] and exhibit a reduced response to LSS when compared with $\alpha\beta\gamma$ channels. Studies of chimeras composed of human α and δ subunits indicated that nonconserved residues within their pore-forming regions accounted for the differences in their response to LSS [71]. Althaus and co-workers performed single channel recordings of oocytes expressing rat or frog ENaC using an outside-out patch configuration, and showed a clear increase of channel P_o in response to a shear force of ~ 0.2 dynes/cm² [69]. In agreement with these observations, flow-dependent increases in Na^+ transport were not prevented by disrupting membrane trafficking, suggesting tubular flow did not alter the channel density at the apical membrane [73].

ENaCs are exposed to a variety of physical forces, including hydrostatic pressure and shear stress. Two models have been proposed to explain how mechanosensitive channels respond to external forces. The 'bilayer model' proposes that channels are gated by membrane tension or deformation induced by mechanical forces. The 'tethered model' proposes that mechanical forces are transmitted directly to a channel through internal and external proteins

that are tethered to the channel [80]. Carattino et al. undertook the task to test the bilayer model and modified membrane properties by changing the temperature or the lipid compositions [81]. Flow-dependent activation of mouse ENaC was altered by changes in temperature, which did not affect the lipid order of oocyte membranes. In addition, the response of ENaC to flow was not altered by cholesterol depletion or the addition of exogenous cholesterol to the oocytes, suggesting that ENaC mechanosensitivity is not dependent on sensing the membrane deformation or tension induced by flow [81]. A subsequent study by Staruschenko's group reported that flow-dependent activation of mouse ENaC in CHO cells was not altered by the disruption of actin cytoskeleton [67]. These studies suggest that both the bilayer model and the tethered model are not relevant for flow-dependent ENaC activation, and raise the possibility that the large, highly structured extracellular regions of ENaC subunits function as mechanosensors. In contrast, mechanisms of mechanosensing in related channels found in *C. elegans* may be more complex, as a tethered model has been proposed to depict their mechanosensitive properties. MEC-4 and MEC-10 are thought to form the core of the mechanosensitive channel complex in *C. elegans* touch receptor neurons. Accessory proteins (MEC-2 and MEC-6) and cytoskeleton components contribute to the channel's mechanosensing properties, including the intracellular tubulins (MEC-12 and MEC-7) and extracellular EGF/Kunitz repeat proteins (MEC-1 and MEC-9) and collagen (MEC-5) [82]. At present, there is no evidence that a similar signal-transducing complex is responsible for ENaC mechanosensitivity.

MULTIPLE CHANNEL DOMAINS PARTICIPATE IN ENaC's RESPONSE TO SHEAR STRESS

Within the extracellular region, the greatest sequence divergence within members of the ENaC/degenerin family is in the finger domain [5, 6, 9]. Our homology model of the finger domain of the α subunit of mouse ENaC has a small anti-parallel β -sheet interfacing with an α helix, forming a "flap" structure (Figure 1) [9, 83]. This peripheral flap is absent in the resolved ASIC1 structure, and we have examined whether the flap structure has a role in facilitating the sensing of external mechanical forces, such as LSS. Trp substitutions at multiple sites within the flap structure altered the response of ENaC to LSS [83]. Disrupting the flap by deleting residues that form the proposed β -sheet led to slower channel activation by LSS. These results suggest that residues within the proposed flap participate in the LSS response [83].

The thumb domain in the resolved structural of ASIC1 is a rigid cylinder-like structure with a loop at its base that is in proximity to the channel pore. Within this loop, a Trp residue has been proposed to couple movements within the extracellular region elicited by acidification to the transmembrane domains [6, 84]. A conserved Tyr residue is present at this site in the three ENaC subunits. We found that mutations at selected sites within this loop in each of the three subunits altered the magnitude or kinetics of ENaC activation by LSS [85], again suggesting that this region has a role in ENaC's response to mechanical forces.

As ENaC activation by shear force reflects a change in channel gating, residues within the pore likely undergo a conformational change in response to LSS. The role of the pore region in channel gating by LSS was examined in channels bearing individual Cys substitutions in the tract S576–S592 of mouse α ENaC [79]. The kinetic characteristics of the channel's response to LSS were altered by Cys substitutions at multiple sites within the pore-forming region of the α subunit, suggesting that residues in the pore participate in the sensing and/or transduction of the mechanical stimulus that results in channel activation [79].

While it is possible to identify sites where substitutions alter the LSS response, it is difficult to ascertain whether these sites are directly involved in flow sensing, or are involved in

transmitting LSS-induced conformational changes to the channel gate. One approach we have taken to identify likely sites that are involved in allosteric conformational changes is to determine whether substitutions alter the response of channels to multiple extracellular factors that modulate channel activity. For example, ENaCs are inhibited by external Na^+ presumably through Na^+ binding to sites within the extracellular region that have not yet been defined [5]. We found a strong correlation between the effects of mutations within the loop at the base of the thumb domain on the effects of LSS and external Na^+ on channel activity [85]. In contrast, we did not observe a correlation with mutations within the peripheral flap in the finger domain [83]. This divergence in the effects of LSS and external Na^+ on channels bearing substitutions within the finger domain suggests that different sites within the finger domain may have roles in sensing LSS and external Na^+ . We suggest that the presence of shear force induces movements at sites within the periphery of the channel, such as the flap within the finger domain, which induces conformational changes within regions that are in proximity to the pore, including the loop at the base of the thumb domain, that is transmitted to the transmembrane helices where the pore and gate reside (Figure 1) [5].

SUMMARY

There is a growing body of evidence that ENaCs are regulated by external factors that affect channel gating, presumably through conformational changes in the extracellular regions of ENaC subunits that are transmitted to the channel gate and pore. Proteases and shear stress are two of these external factors that regulate ENaC. Proteases activate ENaC under a number of different physiologic or pathophysiologic settings, such as volume depletion, aldosterone administration, proteinuria and inflammation. Specific protease inhibitors that prevent ENaC activation by proteases in specific disease states may provide new approaches to disorders associated with ENaC activation. In addition to its expression in epithelia, ENaC subunits are expressed in vascular beds and are thought to have a role modulating vascular tone associated with changes in vascular volume [86-89]. Novel reagents that prevent ENaC activation by shear stress or hydrostatic pressure could dampen flow-dependent K^+ secretion in the distal nephron, as well as dampen the effects of ENaC on vascular tone.

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Figure 1. Model of the extracellular and transmembrane (TM) regions of the α subunit of ENaC. Defined domains in the extracellular region are highlighted (finger, thumb, palm, β -ball, knuckle). Proposed movements induced by external factors such as LSS are indicated by arrows.

Figure 2. Model of the inhibitory tract (arrow) at the interface of the finger and thumb domains. Regions within the finger and thumb domains containing residues predicted to interact with the inhibitory tract are highlighted in a darker shade of grey (blue in the online version). The inhibitory tract constrains movement at a finger-thumb interface, favoring the closed state [33]. Release of the inhibitory tract by proteases activates the channel.