Regulation of epithelial Na\(^+\) channels by aldosterone: Role of Sgk1

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Summary

1. The epithelial sodium channel (ENaC) is tightly regulated by hormonal and humoral factors including cytosolic ion concentration, glucocorticoid and mineralocorticoid hormones. Many of these regulators of ENaC control its activity by regulating its surface expression via Nedd4-2.

2. During the early phase of aldosterone action, Nedd-2-dependent downregulation of ENaC is inhibited by the serum and glucocorticoid-dependent protein kinase, Sgk1.

3. Sgk1 phosphorylates Nedd4-2. Subsequently, phosphorylated Nedd4-2 binds to the 14-3-3 protein and hence diminishes binding of Nedd4-2 to ENaC.

4. Nedd4-2 is also phosphorylated by protein kinase B (Akt1). Both Sgk1 and Akt1 are part of the insulin-signalling pathway that increases transepithelial Na\(^+\) absorption by inhibiting Nedd4-2 and activating ENaC.

Introduction

Epithelial sodium channels (ENaC) are Na\(^+\) transport proteins expressed in the kidney, colon, lung and ducts of the sweat and salivary glands. In these tissues, ENaC facilitates Na\(^+\) absorption. Its function in the kidney and colon is essential for the regulation of Na\(^+\) and fluid homeostasis, particularly in fine-tuning total body Na\(^+\) absorption during volume and osmotic challenges. ENaC is a heteromultimeric protein comprising three homologous subunits, α-, β- and, γ-ENaC, each of which has short cytosolic N and C termini, two membrane-spanning domains and a large extracellular amino acid loop. Although unanimous agreement on the subunit composition of ENaC expressed at the surface of the cell membrane has not yet been reached, it is generally accepted that a functional ENaC complex incorporates all three ENaC subunits. At the cell membrane, the activity of ENaC allows passive movement of Na\(^+\) from the fluid bathing the apical cell membrane of the epithelium along the electrochemical gradient into the cytoplasm. Na\(^+\) absorption via this process (see Figure 1) is driven by activity of the Na\(^+\)/K\(^+\)-ATPase in the basolateral membrane, an active pump that maintains Na\(^+\) concentration in the cytosol at a low level. An additional consequence of Na\(^+\)/K\(^+\)-ATPase activity is the generation of an electrochemical gradient which favours movement of Na\(^+\) from the luminal fluid across the apical membrane into the cell through ENaC. Accordingly, the activity of the Na\(^+\)/K\(^+\)-ATPase is not only critical to the whole transport process but also a limiting factor for the rate at which Na\(^+\) can be transported across the apical membrane.

![Figure 1. Diagram showing a model for Na\(^+\) absorption via ENaC. The luminal plasma membrane contains the amiloride-sensitive Na\(^+\) channels. The electrochemical driving force for Na\(^+\) absorption from the luminal fluid into the cell is generated by activity of the Na\(^+\)/K\(^+\)-ATPase in the basolateral membrane that keeps cytosolic Na\(^+\) concentration low. In this model, Cl\(^-\) ions are transported via Cl\(^-\) channels expressed in both the apical and basolateral membrane.](https://example.com/figure1.png)

It is well established that the activity of ENaC in the distal nephron and the distal colon is upregulated by the mineralocorticoid aldosterone released from the adrenal zona glomerulosa. The aldosterone sensitivity of ENaC makes activity of the channel in these tissues important to the regulation of overall Na\(^+\) and fluid homeostasis and, hence, in the regulation of blood pressure. Consequently, dysfunction of ENaC has been implicated in abnormality of blood pressure regulation and in disorders of the regulation of the thickness of the fluid layer bathing the lung surface.

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ENaC surface expression: the role of ubiquitin-protein-ligases

In aldosterone-sensitive tissues, expression of ENaC is exclusive to the apical membrane of the surface epithelial cells. During biosynthesis, only a small portion of newly synthesized ENaC subunits are processed to the apical cell surface. This inefficiency in the assembly of newly formed ENaC subunits into functional channel complexes leads to the majority of newly synthesised ENaC subunits being targeted for degradation. After it reaches the cell surface, the level of expression of ENaC in the plasma membrane is regulated, allowing activity of the channel to be kept in accordance with the rate of Na⁺ absorption that is required to maintain normal Na⁺ homeostasis. Surplus ENaC proteins are removed from the membrane by an internalisation mechanism that involves activity of Nedd4, a HECT (homologous to E6-AP C-terminus) domain E3 ubiquitin-protein ligase, or its homologue Nedd4-2, both of which directly interact with the channel. This role of Nedd4 and Nedd4-2 is known to be critical to the rapid adaptation of transepithelial Na⁺ transport in response to changes in the cellular ionic environment. A series of studies from our laboratory have demonstrated that both Nedd4 and Nedd4-2 are key mediators of Na⁺ feedback regulation of ENaC, cellular mechanism that downregulates the activity of ENaC in response to increases in intracellular Na⁺ concentration. This Nedd4/Nedd4-2-dependent mechanism might be of physiological importance in coordinating the activity of ENaC and the basolateral membrane Na⁺/K⁺ ATPase, thereby preventing accumulation of intracellular Na⁺ to a toxic level.

To regulate ENaC, a direct interaction between the ubiquitin protein-ligases and the channel is required. Nedd4 and Nedd4-2 bind, via their WW domains, to the proline-rich PY motifs (PPxYxxL) located in the C-terminus of the β- and γ-ENaC subunits. This binding, in turn, leads to polyubiquitination of the channel and promotes its internalisation. It is noteworthy that the contribution of Nedd4-2 to ENaC regulation appears to be greater than that of Nedd4, hence the role of Nedd4-2 as a regulator of ENaC has recently been a subject of considerable interest. Of the four WW domains present in mouse collecting duct cells, in addition, in Xenopus oocytes and in Chinese hamster ovari cells, ENaC activity was found to significantly increase if activity of dynamin, a critical component of clathrin-mediated endocytosis, was suppressed. Furthermore, in mouse collecting duct cells, overexpression of epsin, a protein that has both clathrin-interacting motifs and ubiquitin-binding motifs that are capable of binding ubiquitinated ENaC, decreased ENaC activity.

Although the cellular mechanisms that underlie ENaC internalisation remain to be fully elucidated, it is generally accepted that Nedd4-2-mediated internalisation of ENaC is of great physiological importance for keeping basal activity of the channel in the distal kidney at an appropriate level. This physiological significance of Nedd4-2 is apparent in the situation where interaction between ubiquitin protein-ligases and ENaC is disrupted, as in Liddle’s syndrome, an autosomal dominant form of salt-sensitive hypertension with hypokalemic alkalosis and low renin and aldosterone activity. Liddle’s syndrome is caused by genetic mutations that delete the cytoplasmic termini of β- or γ-ENaC, rendering ENaC incapable of interacting with Nedd4-2 due to loss of the required binding sites. Loss of the PY motifs from β- and γ-ENaC C-termini results in increased cell surface expression of the channel. Conversely, overexpression of exogenous Nedd4-2 decreases both ENaC surface expression and channel activity. Together these findings emphasise the significance of Nedd4-2 for the regulation of membrane expression of ENaC. It remains unknown, however, whether Nedd4-2 is capable of regulating ENaC internalisation alone, or if it also influences trafficking of immature ENaC to the cell surface. Recently a study indicates that Nedd4-2 only interacts with mature ENaC at the cell surface, which may imply that activity of Nedd4-2 is limited to the regulation of channels that are already expressed at the cell membrane, and is not involved in the regulation of the abundance of immature ENaC subunits in the cytosolic compartments.

Sgk1 mediation of the aldosterone effect on ENaC

Aldosterone is a potent activator of ENaC activity in the distal nephron, where it increases both biosynthesis and activity of Na⁺ transporters at the cell membrane. The natriferic effect of aldosterone can be viewed as having two phases. An initial effect of aldosterone on ENaC activity is apparent within 30 minutes following exposure of the target tissues to the hormone. During this initial phase, total cellular expression of ENaC and of Na⁺/K⁺-ATPase remain unchanged, whereas the cell membrane abundance of both transporters was notably elevated. Translocation of pre-existing ENaC subunits from cytosolic compartments to the apical cell surface and/or the reduction of the rate of ENaC internalisation are the most likely contributors to the rapid increase in Na⁺ absorption during this period. A subsequent increase in transepithelial Na⁺ absorption in aldosterone-targeted tissues occurs 3-6 hours after exposure to the hormone. This later effect of aldosterone is sustained,
In mammalian kidney, where its expression can be detected from the thick ascending limb of Henle to the cortical collecting tubule, and this expression pattern of Sgk1 might suggest that the mechanism by which Sgk1 is activated and its physiological roles could differ substantially from those of the other two Sgk isoforms. In addition to serum and glucocorticoids, aldosterone on Na absorption during this late period of aldosterone action. Nevertheless, the susceptibility of Sgk1 to different regulators, and the variety of transport proteins that the kinase might regulate, suggest that Sgk1 is an important convergence point for coordinating multiple ion transporter activities by various physiological stimuli in both the normal and pathological states.

Among Sgk proteins, Sgk1 and Sgk3, but not Sgk2, are known to be potent activators of ENaC. Even so, Sgk1 is the only Sgk isoform that is robustly expressed in mammalian kidney, where its expression can be detected from the thick ascending limb of Henle to the cortical collecting tubule, and this expression pattern of Sgk1 overlaps with that of ENaC in the distal part of the nephron. Investigation of Sgk1 as an important mediator of aldosterone on Na absorption began with two studies which demonstrated that transcription of this kinase in the kidney is regulated by aldosterone, and that, when coexpressed in Xenopus oocytes, Sgk1 significantly enhances the activity of ENaC. Additionally, aldosterone is essential for the maintenance of basal Sgk1 activity in the kidney. Over half of Sgk1 expression in the distal nephron of normal rat is maintained by serum aldosterone. Furthermore, aldosterone treatment rapidly activates transcription of Sgk1 mRNA along the entire length of the aldosterone-sensitive distal nephron and concurrently increases expression of α-ENaC and Na absorption in the same nephron segment. In addition to its effect on ENaC, Sgk1 also activates Na transport across the basolateral membrane by drastically increasing both expression and activity of the Na+/K+-ATPase. These properties allow the kinase to balance apical entry of Na via ENaC with basolateral exit of the ion via the Na+/K+-ATPase. Given that concurrent increases of Na transport mechanisms in both membrane domains are required to maintain Na absorption at a higher level, the ability to simultaneously control both ENaC and Na+/K+-ATPase makes Sgk1 highly effective in modulating net Na absorption. It should, however, be noted that, despite the continuing presence of aldosterone, Sgk1 mRNA and protein levels in A6 amphibian kidney cell lines return to near basal levels within 24 hours following aldosterone treatment. This unsustained activation of Sgk1 activity by aldosterone might suggest that activity of this kinase only accounts for mediation of the early effect of the hormone on Na absorption.

Sgk1 increases ENaC activity by inhibiting Nedd4-2

The stimulatory effect of Sgk1 on ENaC activity can, by large, be explained by its stimulatory effect on the rate of internalisation of the channel. The striking opposition between the influences of Sgk1 and of Nedd4-2 on the abundance of ENaC at the cell membrane has inspired numerous investigations into the relative roles of these two effector proteins in the cellular pathways which regulate ENaC membrane expression. Evidence from several laboratories has shown that phosphorylation of Nedd4-2 is an important step by which the kinase modulates the activity of ENaC. At the molecular level, Sgk phosphorylates Nedd4-2 at three putative sites, and Thr367. Phosphorylation of these residues decreases the ability of Nedd4-2 to interact with and ubiquitinate ENaC, thereby disrupting Nedd4-2-dependent downregulation of the channel. Phosphorylation of Nedd4-2-dependent may be integral to the mechanism whereby several regulators of ENaC exert their stimulatory effects on the activity of the channel. For instance, aldosterone increases phosphorylation of Nedd4-2 in both rat kidney and mpkCCD Cl cultured collecting duct cells. Nedd4-2 is also a phosphorylation target of cAMP-dependent protein kinase (PKA), and phosphorylation of Nedd4-2 by PKA may be involved in the mechanism by which vasopressin upregulates Na transport via ENaC. Conversely, constitutively phosphorylated Nedd4-2 may have an adverse effect on overall Na and fluid homeostasis. A naturally occurring mutant of Nedd4-2, Nedd4-2<sup>3558</sup> has
recently been identified in end-stage renal disease patients with arterial hypertension.\(^7\) This mutated form of Nedd4-2 exhibits a strong basal phosphorylation level and a weaker inhibitory effect on ENaC than the wild-type form. Although phosphorylation by Sgk1 does weaken the ability of Nedd4-2 to bind to ENaC, phosphorylated Nedd4-2 reciprocally inactivates Sgk1 activity by increasing ubiquitination of the kinase, leading to degradation of Sgk1 in a 26S proteosome-dependent manner.\(^2\) Such a feedback mechanism might allow Sgk1 activity to be self-regulated, which would permit fine-tuning of ENaC activity in response to activation by hormones such as aldosterone and glucocorticoids.

Nedd4-2 might not be the only phosphorylation target of Sgk1 that is involved in ENaC regulation. It has recently been reported that in *Xenopus* oocytes, Sgk1 has no effect on C-terminal truncated ENaC lacking Nedd4-2 binding sites, and that the effect of Sgk1 is mediated via Ser\(^621\) on the C-terminal of αENaC.\(^7\) This finding prompts the speculation that direct phosphorylation of ENaC might be involved in the actions of Sgk1. On the other hand, expression of a constitutively active Sgk1 mutant (Sgk1\(^{S425D}\)) was without effect on the phosphorylation level of ENaC subunits in A6 amphibian kidney cells.\(^7\) The discrepancy between these two findings could be attributed to either the inability of Sgk1\(^{S425D}\) to exert its kinase activity on ENaC or to the failure of the oocyte expression system to reflect the real physiological role of the kinase on the channel. Nevertheless, the notion that Sgk1 may directly phosphorylate and activate ENaC is interesting and deserves further rigorous investigation.

**Phosphorylated Nedd4-2 interacts with 14-3-3**

Phosphorylated Nedd4-2 binds preferentially to 14-3-3,\(^79,80\) a dimeric acidic protein ubiquitous in eukaryotic cells.\(^81,82\) Binding of phosphorylated Nedd4-2 to 14-3-3, in turn, prevents interaction between the WW domains of Nedd4-2 and the PY motifs of ENaC, while also preventing the dephosphorylation of Nedd4-2.\(^80\) It would thus appear that 14-3-3-dependent inhibition of Nedd4-2 activity plays a key role in regulation of Sgk1-dependent upregulation of ENaC.\(^79\) There are several lines of evidence that support this notion. These include the observation that overexpression of a Nedd4-2 mutant that is sensitive to Sgk1 phosphorylation, but which cannot interact with 14-3-3, dampened the stimulatory effect of Sgk1 on ENaC activity.\(^80\) In addition, knock-down of 14-3-3 expression, using a 14-3-3-specific siRNA, decreased ENaC expression, increased the association between ENaC and Nedd4-2 and abrogated the stimulatory effect of aldosterone on ENaC.\(^83\) Furthermore, 14-3-3 also plays an important role in the mechanism by which aldosterone increases ENaC activity. Recent evidence indicates that aldosterone strongly increases expression of 14-3-3 mRNA without affecting the level of total Nedd4-2 expression.\(^83\) Concomitantly, aldosterone decreases the association between ENaC and Nedd4-2 and increases the association between the free phosphorylated Nedd4-2 and 14-3-3.\(^83,84\) Together, activation of Sgk1, phosphorylation of Nedd4-2 and recruitment of 14-3-3, leading to the depletion of Nedd4-2 activity, may be a key mechanism employed by aldosterone to increase Na\(^+\) absorption via ENaC (Figure 2).

### Possible roles of other kinases and future directions

Recent studies have shown that downregulation of Nedd4-2 activity is a critical step in the signalling pathway by which aldosterone exerts its early effects on sodium transport. Most studies have focused on the role of Sgk1 as the kinase in the aldosterone signalling pathway that phosphorylates Nedd4-2 and renders it ineffective. Nevertheless, it is surprising that mice with Sgk1 knockout phenotype (Sgk1\(^{-/-}\)) show no difference in renal Na\(^+\) excretion compared to wildtype (Sgk1\(^{+/+}\)) when the animals were maintained on a normal Na\(^+\) diet. Such observations can be interpreted as indicating either that the activity of Sgk1 is not critical for maintaining basal activity of ENaC in the distal nephron or that there is a redundant mechanism capable of maintaining normal ENaC activity in the kidney in the absence of Sgk1. An inability of the distal tubule of Sgk1\(^{-/-}\) mice to increase Na\(^+\) retention, however,
became apparent when the mice were challenged with a low Na\textsuperscript{+} diet.

When we consider the possibility that an alternative regulatory protein may act on ENaC in the absence of Sgk1, we need to bear in mind a protein in the Sgk family which is structurally very similar to Sgk1, Akt1, also called protein kinase B, which is a key mediator in the insulin signalling pathway. The catalytic domains of Akt and Sgk1 have over 55\% homology in their amino acid sequence. In addition, Akt1 has conserved residues that resemble the amino acid sequence of a domain in Sgk1 required for its activation.\textsuperscript{44} Furthermore, both Sgk1 and Akt1 are activated by PI3K/PDK1,\textsuperscript{85} key mediators of the effects of aldosterone and insulin on ENaC. The surprising failure of overexpression of a constitutively active mutant of Akt1 to elevate basal activity of ENaC\textsuperscript{86} has prompted the suggestion that Akt1 may not be involved in the regulation of ENaC either under basal conditions or during stimulation with insulin and aldosterone. Current data from our laboratory, however, dispute this notion. In agreement with previous reports, we found that the constitutively active mutant Akt1 not only failed to increase ENaC activity, but also inhibited the channel.\textsuperscript{87} We found, however, that overexpression of a wild-type Akt1 increased ENaC activity.\textsuperscript{87} The stimulatory effect of the wild-type Akt1 on ENaC was also confirmed by the finding that inhibition of Akt1 activity, either by a small-interference RNA directed against Akt1 or by a dominant negative Akt1, decreased the activity of ENaC.\textsuperscript{87} In addition, we found that activation of ENaC by Akt1 requires phosphorylation of Nedd4-2 and is a critical component of the insulin signalling pathway that upregulates ENaC activity.\textsuperscript{87} Together, these data suggest that Akt1 is an important activator of ENaC. Nonetheless, whether Akt1 alone can maintain activity ENaC at a normal level in the absence of Sgk1, such as occurs in Sgk1(-/-) mice, remains to be investigated.

Sgk1 exerts its effects on ENaC by downregulating the inhibitory effect of Nedd4-2 on the channel in a phosphorylation-dependent manner. Interestingly, Nedd4-2 also contains phosphorylation sites for the G-protein receptor kinase, Grk2.\textsuperscript{88} A known regulator of ENaC that increases ENaC activity by inhibiting feedback downregulation of the channel in response to increases in cytosolic Na\textsuperscript{+} concentration, as a consequence of phosphorylating the β-ENaC C-terminal.\textsuperscript{27} Nevertheless, the presence of consensus phosphorylation sites for Grk2 on Nedd4-2, suggests that phosphorylation of Nedd4-2 by Grk2 might, at least in part, have some role in modulating ENaC activity. This possibility still remains to be explored.

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