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# Mechanisms of Aldosterone's Action on Epithelial $Na^+$ Transport

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**Abstract.** Aldosterone maintains total organism sodium balance in all higher vertebrates. The level of sodium reabsorption is primarily determined by the action of aldosterone on epithelial sodium channels (ENaC) in the distal nephron. Recent work shows that, in an aldosterone-sensitive renal cell line (A6), aldosterone regulates sodium reabsorption by short- and long-term processes. In the short term, aldosterone regulates sodium transport by inducing expression of the small G-protein, K-Ras2A, by stimulating the activity of methyl transferase and S-adenosyl-homocysteine hydrolase to activate Ras by methylation, and, possibly, by subsequent activation by K-Ras2A of phosphatidylinositol phosphate-5-kinase (PIP-5-K) and phosphatidylinositol-3-kinase (PI-3-K), which ultimately activates ENaC. In the long term, aldosterone regulates sodium transport by altering trafficking, assembly, and degradation of ENaC.

## Introduction

Fifty years ago Hans Ussing described the mechanism by which sodium is actively transported across frog skin [49]. While the fundamental mechanism was described in Ussing's initial paper, in the ensuing 42 years, an enormous amount of effort was invested in determining the specifics of the mechanism and, possibly more importantly, the mechanisms by which sodium transport can be regulated (for reviews, *see* [5, 19]). In this period, which might be termed the "pre-molecular era", a surprisingly detailed picture of the sodium transport mechanism was developed. Ussing had originally suggested that sodium transport required passive, energetically downhill movement into the cell across the apical membrane of epithelial cells with active, energetically uphill movement out of the cell at the basolateral membrane mediated by the Na,K-ATPase. Although the Na,K-ATPase was ubiquitously present in virtually all multicellular organisms (and consequently received significant research attention), the apical sodium entry mechanism was thought to be unique to sodium-transporting epithelial cells. By the early 1990s many characteristics of this apical entry pathway had been described: the pathway was an ion channel that was highly selective for sodium and lithium over other cations and each channel had a low individual or unit conductance; the channel was sensitive to low concentrations of amiloride and contained a single sodium binding site; and the channel was regulated by several cellular signaling pathways, the most important of which appeared to be increased activity in response to the steroid hormone, aldosterone. In much of this work, Ussing and his colleagues provided seminal insight [14, 18, 47, 48, 50]. Despite the large amount of information gathered in the "pre-molecular era", progress since the initial cloning of the sodium channel subunits by Canessa and Rossier [9, 10] has led to an enormous increase in our understanding of the structure, function and regulation of the epithelial sodium channel (ENaC). This article will concentrate on one aspect of ENaC regulation: that associated with the steroid hormone, aldosterone.

## Regulation of $Na^+$ transport by aldosterone

Sodium transport across the tight epithelia of $Na^+$ reabsorbing tissues such as the distal nephron and colon is the major factor determining total-body $Na^+$ levels, and thus, long-term blood pressure. Regulation of sodium reabsorption occurs mostly in the distal nephron where reabsorption is a two-step process as originally described by Ussing for frog skin. First, sodium enters renal cells from the luminal compartment through ENaC in the apical membrane before it is actively transported out of the cell by the basolateral Na,K-ATPase. ENaC is usually the rate-

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limiting step for $Na^+$ transport and, therefore, the target of many hormones that regulate $Na^+$ balance. The mineralocorticoid, aldosterone, is the major hormone that specifically increases $Na^+$ influx at the apical membrane. The increase occurs in two phases: an initial phase, which increases transport 4- to 6-fold in the first 2 to 6 hours and a late phase, which requires 12–48 hours and increases transport another 3- to 4-fold. The mechanisms for the early and late phases appear to be different. Aldosterone, like other steroid hormones, enters target cells and binds to cytosolic, mineralocorticoid receptor complexes. After some rearrangement, the aldosterone-bound receptor acts as a DNA-binding protein that targets steroid response elements on genetic DNA. Binding to the response elements alters gene expression. Increases in $Na^+$ transport can be measured within 1 hour of exposure to aldosterone, and this increase is dependent on gene transcription and translation, with the gene products generically referred to as aldosterone-induced proteins (AIPs).

Many attempts using a variety of differential screening methods have been made to identify the aldosterone-induced gene products. These attempts have met with varying levels of success, but the relationship between the genes identified and the mechanism by which aldosterone increases $Na^+$ transport is often unclear. Originally, because of the observation that protein synthesis was required for aldosterone to increase sodium transport, it was hypothesized that aldosterone induces ENaC synthesis and insertion. While the evidence from electrophysiological measurements remains somewhat controversial [3, 7, 20], all of the available biochemical evidence suggests that the number of sodium channel proteins in the apical membrane does not change after aldosterone treatment (at least in the first two to four hours when the increase in sodium transport is most dramatic) [15, 21, 22, 27]. More recent evidence also supports the view that the early effects of aldosterone are indirect and that the proteins that are synthesized are modulatory proteins, which convert poorly transporting ENaC in the apical membrane into ones that readily transport sodium (*see* below).

The genes encoding ENaC have been identified and shown to form a heteromultimeric channel protein composed of three homologous subunits called α, β, and γ. Several hypotheses are consistent with an aldosterone-induced change in sodium transport with no change in apparent channel number associated with the short-term actions of aldosterone. The most obvious is that there is a post-translational modification of the channels that increases their open probability. The second is that most or all of the channel subunits are separately present in the apical membrane, but not assembled into functional channels; and that aldosterone promotes the assembly of a channel complex capable of transporting sodium.

The mechanism for the long-term effect of aldosterone to increase sodium transport is also unclear. There is evidence in a variety of tissues that the total cellular amount of ENaC subunits may increase; however, whether this is due to an increase in translation is controversial since changes in message levels are quite variable in different tissues. Therefore, the long-term changes could be due to an increase in translation, an increase in trafficking to the membrane, post-translational modifications of channels in the membrane, or a reduction in degradation of membrane channels.

## The Mechanism for the Short-Term Effects of Aldosterone in A6 Cells

As mentioned above, one obvious possibility for the activation of sodium channels with little or no change in the number is through a post-translational modification that alters the open probability of channels already present in the surface membrane. The problem with this idea was that most of the usual post-translational modifications like phosphorylation seemed to have either no effect on channel-open probability [26] or actually decrease activity [16, 23]. However, other post-translational modifications do appear to change in response to aldosterone and alter sodium channel-open probability.

## Aldosterone-induced Methylation Activates ENaC

Sariban-Sohraby et al. [34] demonstrated that the amount of sodium transport that could be measured in apical membrane vesicles obtained from A6 cells, a sodium-transporting, distal-nephron cell line, was markedly enhanced by prior application of agents that methylate membrane proteins. Since they demonstrated that application of aldosterone also leads to the methylation of membrane protein, their suggestion was that intracellular methyl transferases induced by aldosterone could be responsible for the methylation and, therefore, modulate the ENaC. In subsequent work, Sariban-Sohraby et al. [35] also demonstrated that a 95 KDa protein was methylated in the presence of aldosterone. Since this size is consistent with the size of a glycosolated ENaC subunit some investigators have suggested that the target for methylation is an ENaC subunit [31]. However, the primary sequence of the subunits is difficult to reconcile with this view. Almost exclusively, reversible methylation of signaling proteins occurs only at a very restrictive consensus sequence: the so-called "CAAX" box, a methylated cysteine residue followed by two aliphatic residues, followed by any residue at the C-terminal end of a protein. Even if we presumed that there might be significant C-terminal proteolytic trimming of ENaC, there are no cysteine residues that

meet the criteria for methylation. However, there are other possible cellular targets for aldosterone-induced methylation of ENaC regulatory proteins.

## K-Ras2A Is an Aldosterone-induced Protein

In previous work, investigators from our laboratory, using patch-clamp methods, have shown that methylation of isolated, cell-free patches of membrane increases the activity of ENaC [4]. Therefore, the target for methylation, if it is not a channel subunit itself, must be a membrane-associated protein in close proximity to the channels. In addition, the same work by Becchetti et al. and also work by Sariban-Sohraby et al. [35] showed that methylation-induced activation of ENaC is augmented by cytosolic GTP. Thus, methylation of excised, apical membranes induces ENaC activity similar to the activity produced by aldosterone and the methylation reaction is augmented by G protein stimulation. This is an interesting observation, since, in one of the many attempts to identify proteins differentially expressed by aldosterone, Spindler et al. [36] found an aldosterone-induced increase in the message for the small G protein, K-Ras2A. K-Ras2A contains the consensus sequence for methylation, a CAAX box, and is known to require both GTP and methylation for activity. Recent evidence from our laboratory shows that Ras protein is a target for methylation and that Ras methylation is induced by aldosterone [1, 45]. K-Ras is methylated by an isoprenylcysteine methyl transferase. This enzyme requires the presence of an isoprenyl group on the cysteine of the CAAX box [44]. Therefore, it is not surprising that inhibition of prenylation also blocks the action of aldosterone [6]. It is also interesting that the association of 21 KDa Ras with a 70–75 KDa ENaC subunit would produce a 95 KDa complex similar to that previously described by Sariban-Sohraby [35] that would be both methylated and stimulated by GTP. These results show that K-Ras2A is an aldosterone-induced protein.

## Regulation of Methylation. Is Methyltransferase an Aldosterone-induced Protein?

Even though K-Ras2A is an aldosterone-induced protein, to be active K-Ras2A must be methylated. Therefore, if the magnitude of the aldosterone-induced increase in $Na^+$ transport is to be proportional to the extent of K-Ras2A transcription, then aldosterone must alter methyl-transferase activity to ensure that the rate of Ras methylation does not become the rate-limiting step in the induction of ENaC. This increase in methyl transferase activity could arise either by transcription and translation of new methyl transferase or by activation of pre-existing methyl transferase. We have shown in recent work that the isoprenyl-cysteine-O-carboxy-methyl-transferase responsible for K-Ras2A methylation is not an aldosterone-induced protein, but that the intrinsic activity of the enzyme does increase in response to aldosterone [44]. Therefore, it is important to understand how this methyl transferase is regulated. One way of regulating active, membrane-associated methyl transferases is by controlling the cytosolic concentrations of the end product of methylation, S-adenosyl-homocysteine (SAH). This is the product that is produced when the endogenous methyl donor, S-adenosyl-methionine, transfers its methyl group to a target protein. SAH is a potent inhibitor for all methyl transferases [13]. The primary determinant of cellular SAH concentration is generally not the rate of SAH production by methyl transferases; but, rather, its rate of degradation that is determined by another enzyme, S-adenosyl-L-homocysteine hydrolase. Thus, the extent of protein methylation could be controlled by altering the activity of the SAH hydrolase rather than altering the intrinsic activity of the methyl transferase. Work from our laboratory [1, 42, 44, 45] indicates that aldosterone regulates the activity of both enzymes (isoprenyl-cysteine-O-carboxy-methyltransferase and SAH hydrolase). The increase in the activity of the methyl transferase is an increase in $V_{\max}$ possibly produced by phosphorylation of the transferase. The increase in the activity of SAH hydrolase ensures that the methyl transferase is not inhibited by the additional production of SAH after treatment of cells with aldosterone. How the SAH hydrolase is activated is unclear; however, like the methyl transferase, it is also not activated by additional transcription and translation; that is, it is not an aldosterone-induced protein.

## How Does K-Ras2A Increase the Activity of $Na^+$ Channels

Although recent work from our laboratory has demonstrated a role for K-Ras2A [1, 45], the results do not clearly show the mechanism(s) by which K-Ras activates ENaC. The simplest mechanism would be direct ENaC/K-Ras interaction. However, it is likely that there are other signaling elements that lie between Ras and ENaC (although any such element would have to be closely associated with ENaC in cell-free patches since it is possible to activate ENaC via Ras methylation in such patches). The traditional effector for Ras is Raf kinase. Raf activation leads to activation of the mitogen-activated protein-kinase pathway. However, activation of MAPK pathway appears to inhibit ENaC activity rather than stimulate it [41, 43]. Alternatively, small G proteins like K-Ras also activate lipid kinases [11, 17]. Therefore, it is interesting that one product of phosphoinositol phosphate-5-kinase (PIP-5-K) is 4,5-Phosphoinositol-bis-phosphate ($PIP_2$), which strongly activates ENaC in isolated patches of membrane excised from

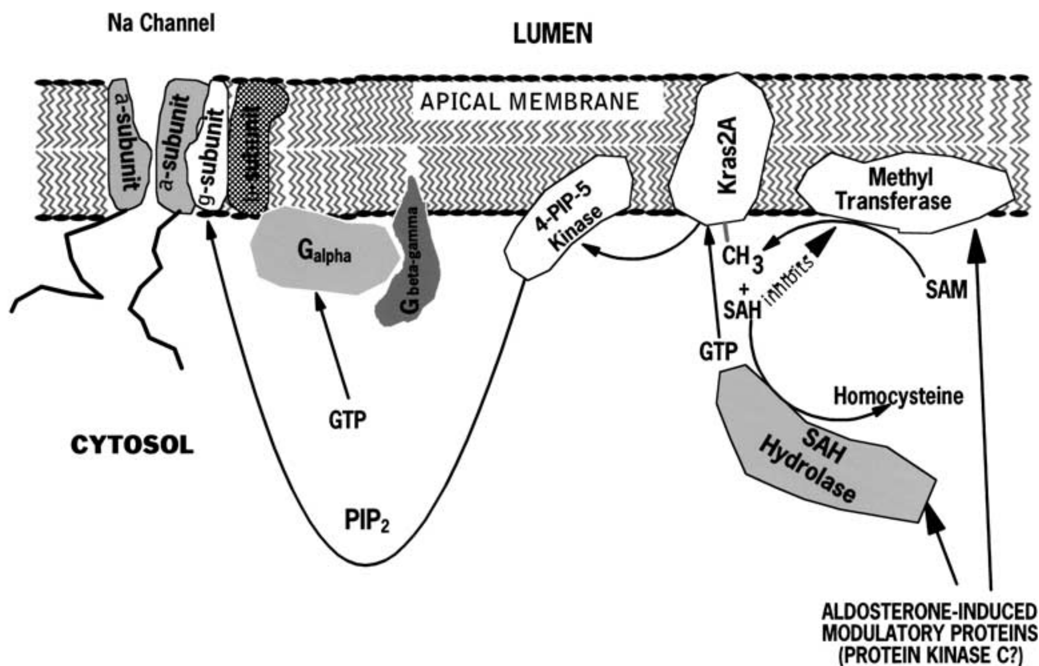


**Fig. 1.** Schematic diagram of the early aldosterone-signaling pathway. The schematic is based on the observations that aldosterone- induced increases in $Na^+$transport require (1) activation of methyl transferase, (2) activation of SAH Hydrolase, (3) new expression of methylated K-Ras2A, and (4) that 4,5-$PIP_2$ strongly activates sodium channels in excised patches of membrane in a GTP-dependent fashion.

A6 cells [53] in a G-protein-dependent fashion. Thus, $PIP_2$ could be the mediator for the short-term action of aldosterone and could be the end result of aldosterone-induced activation of K-Ras2A. The production of $PIP_2$ is interesting for another reason: 4,5-$PIP_2$ is the natural substrate for PI-3-kinase. Ras proteins are also known activators of PI-3-K, which metabolizes 4,5-$PIP_2$ to 3,4,5-$PIP_3$. In addition, inhibition of PI-3-K with LY294002 inhibits the long-term aldosterone-induced increases in $Na^+$ transport [8, 29]. Fig. 1 is a schematic diagram of the possible components of the aldosterone signaling cascade that have been described so far in this article.

## The Mechanism for the Long-Term Effects of Aldosterone in A6 Cells

The observation that PI-3-K is required for aldosterone activation at times longer than 4 hours suggests an alternative mechanism for the activation of ENaC by aldosterone after the initial $PIP_2$-mediated increase in open-probability. The product of PI-3-K, $PIB_3$ activates a variety of proteins that have $PIP_2$-binding domains (also known as pleckstrin homology or PH domains). Notable among these proteins are the phosphatidylinositol-dependent kinases, PDK1/2 [46, 52]. Activation of PDK1/2 is interesting since one of the kinase's phosphorylation targets is another kinase, serum-and-glucocorticoid-dependent kinase (SGK). This kinase is an aldosterone-induced gene [28, 30] and the long-term effects of aldosterone require activation of SGK by phosphorylation [51]. The kinase activity of SGK may target a variety of proteins; however, one target is the ubiquitin ligase, Nedd4. Nedd4 is intimately related to ENaC protein turnover from the surface membrane [24, 32, 33, 37].

## A Specific Ubiquitin Ligase, Nedd4, Is Responsible for Ubiquitination and Subsequent Degradation of Membrane ENaC

For many proteins, including ENaC, ubiquitin conjugation is a prerequisite for membrane internalization and subsequent degradation. In A6 cells, membrane-associated ENaC $\alpha$ and $\beta$ subunits are ubiquitin-conjugated (whether the $\gamma$ subunit is also ubiquitinated has not been determined) and apparently degraded by proteasomes [24]. In A6 cells ubiquitin-conjugation of ENaC subunits requires a specific ubiquitin ligase, Nedd4 (neural-precursor-cell-expressed-developmentally-down-regulated protein). Nedd4 contains an E6-AP carboxyl terminus (Hect) domain that is homologous to other ubiquitin ligases, and three or four WW domains depending on the species, and a calcium/lipid binding domain (CaLB/C2) [39]. Nedd4 was found to have the same localization pattern as ENaC in renal cortical and outer medulla collecting duct principal cells and in airway epithelia [40]. Nedd4 apparently requires direct association with ENaC to produce ubiquitin conjugation. Nedd4 can regulate the number of functional ENaC in the surface membrane since, after Nedd4

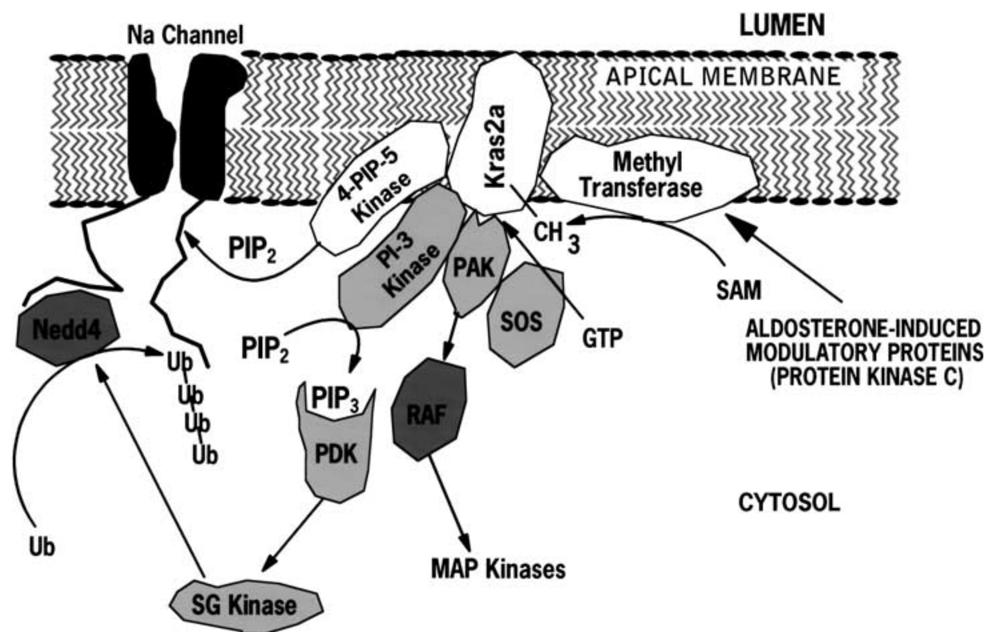


**Fig. 2.** Schematic diagram of the overall aldosterone signaling pathway. The schematic includes both the early activation process (represented in Fig. 1), which involves K-Ras activation of 4-PIP-5-kinase and production of $PIP_2$, which activates channels, well as a proposed model for the late aldosterone responses in which PI-3-kinase is activated by K-Ras to metabolize $PIP_2$ to $PIP_3$. $PIP_3$ activates PDF, which phosphorylates SGK. SGK phosphorylates Nedd4. Phosphorylation of Nedd4 reduces its affinity for ENaC subunits and, thereby, reduces ENaC-subunit ubiquitin conjugation. In the absence of ubiquitin conjugation, ENaC subunits are not internalized and degraded and accumulate in the surface membrane where they contribute to the aldosterone-induced increases in sodium transport.

synthesis was blocked with anti-sense oligonucleotides in native renal cells (A6), there was an increase in the transepithelial current. These results imply that Nedd4 is the ubiquitin ligase for ENaC in native renal cells.

## ENaC Degradation in A6 Epithelial Cells

In A6 cells, proteasome inhibition increases the total cellular amount of all ENaC subunits [24]. Also, the half lives of all subunits in the total cellular pool is relatively short (1–3 hrs) and increases several-fold after proteasome inhibition [24, 38]. In A6 cells, proteasome inhibition caused an increase in amiloride-sensitive transepithelial current. The increase in amiloride-sensitive transepithelial current induced by inhibiting proteasome activity is associated with an increase in the apical density of sodium channels measured by patch-clamp methods, an increase in number of ENaC subunits that can be surface labeled with biotin [25] and an increase in the ubiquitination of ENaC in the surface membrane. Also, the half-lives of membrane-associated (biotinylated) $\beta$ and $\gamma$ ENaC subunits increased from 3.7 hours for $\beta$ and 7.5 hours for $\gamma$ subunit to greater than 24 hours when proteasomes were inhibited [24]. The half-life of the $\alpha$ subunit may also increase after proteasome inhibition, but the normal half life for $\alpha$ subunits at the surface of native cells is at least 42 hours [22] and, therefore, an additional increase after proteasome inhibition is difficult to measure [24].

## Aldosterone Regulation of ENaC Degradation Rate in A6 Cells

Aldosterone alters the amount of ENaC subunits on the apical surface of A6 cells even in the absence of any significant change in message levels for any of the subunits [2, 27]. This would seem to imply that aldosterone can alter ENaC degradation. In A6 cells, aldosterone increases the amount and activity of SGK [2, 12, 28]. One target of this kinase is Nedd4, which, when phosphorylated has a lower affinity for binding to ENaC subunits (O. Staub, in press). Fig. 2 provides a schematic view of possible mechanisms for aldosterone action.

Thus, aldosterone, the hormone primarily responsible for altering epithelial Na transport, apparently accomplishes the increase in two ways. Initially, as a rapid response, aldosterone promotes the production of $PIP_2$, which rapidly increases the activity of ENaCs that are already in the surface membrane. Later, aldosterone apparently increases the number of functional channels in the surface membrane, at least in part, by reducing the degradation rate of ENaC and allowing an increase in the surface membrane pool of functional ENaC.

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