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ALDOSTERONE ACTION IN EPITHELIA: REGULATED GENE PRODUCTS

Regulation of the epithelial Na^+ channel by aldosterone: Open questions and emerging answers

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Regulation of the epithelial Na^+ channel by aldosterone: Open questions and emerging answers. Aldosterone is the principal adrenal steroid controlling Na^+ retention in amphibians and mammalians. It acts primarily by increasing the apical Na^+ permeability through activation of the epithelial Na^+ channel (ENaC). The cellular events mediating the hormonal action are mostly unknown. Early studies have provided evidence that the hormone functions to activate or translocate pre-existing channels by a yet undefined mechanism. In addition, enhanced de novo channel synthesis appears to take place as well. The molecular cloning of the three ENaC subunits has provided new powerful tools for testing and confirming this hypothesis, as well as for characterizing mechanisms by which ENaC is regulated. Another important development is the recent identification of several cDNAs corresponding to aldosterone-induced and suppressed mRNAs. The study of these genes and their putative interactions with ENaC is likely to provide important clues to the mechanisms by which aldosterone controls the apical Na^+ permeability of tight epithelia. This article reviews recent developments in the field that may lead to the elucidation of the mechanisms by which the hormone controls Na^+ transport.

The mineralocorticoid aldosterone is the major hormone controlling Na^+ retention in vertebrates. It acts by enhancing Na^+ reabsorption across tight epithelia such as kidney collecting duct and distal colon. Early studies have established that the hormone functions to increase both the passive luminal entry of Na^+ into epithelial cells and its active extrusion into the blood [reviewed in 1–4]. These take place in different time scales and are mediated by different cellular events. Under most conditions, Na^+ transport in distal nephron is limited by the rate of Na^+ entry through the amiloride-blockable epithelial Na^+ channel (ENaC). Hence, the luminal action of aldosterone is considered to be the major one.

An increase in the apical, amiloride-blockable Na^+ permeability is apparent 30 to 90 minutes after applying aldosterone, and it reaches a maximal value several hours later. It is primarily caused by an increase in the number of "electrically detectable" Na^+ channels in the apical surface (N), with no change in their open probability (P_0) or single channel current (i) [5–9]. Thus, the hormone acts either by translocating new channels to the apical surface or by activating a pool of "silent" apical channels that do not contribute to P_0. This response is fully blocked by transcription or translation inhibitors, suggesting that it is mediated by a "classic" steroid mechanism through alterations in gene expression. Nongenomic effects of aldosterone have been described as well and may, in principle, contribute to its natriferic action [10, 11].

Early studies have provided substantial evidence that the apical action of aldosterone is not likely to represent enhanced transcription/translation of the channel protein [reviewed in 1, 2, 12, 13]. Later, it was realized that the hormonal stimulation of Na^+ channel activity is a more complex response and may involve both the activation (or translocation) of pre-existing channels and the enhanced transcription/translation of channel subunits [2, 3, 14–16]. The first response is thought to provide a major contribution to the initial increase in channel activity, whereas the second seems to be important during chronic exposure to the hormone. A key unresolved issue is the identity of aldosterone-induced (or suppressed) proteins and the nature of events relating them to an increase in the apical Na^+ permeability.

The molecular cloning of ENaC and its identification as the luminal amiloride-blockable conductance in tight epithelia mark a turning point in the field [17–20]. This channel is composed of three homologous subunits denoted α, β, and γENaC and defines a new gene family, which includes several other epithelial and neuroneal cationic channels, as well as *C. elegans* genes mediating mechanical stress [21–23]. The expressional cloning of ENaC has enabled the development of new powerful tools to study its modulation by aldosterone. These include specific antibodies, cDNA probes, and useful expression

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systems in which interactions between ENaC and putative aldosterone-induced effectors can be assessed. In addition, it has provided clear-cut proof for the central role of this channel (and its mineralocorticoid regulation) in whole-body electrolyte homeostasis. This is apparent from the study of genetic diseases caused by mutations in ENaC subunits, as well as the phenotypic analysis of ENaC knockout mice [reviewed in 21, 23–26].

The current article briefly reviews the recent developments in the regulation of ENaC by aldosterone, which appears to pave the way for the elucidation of the mechanisms by which it controls the apical Na^+ permeability.

EFFECTS OF ALDOSTERONE ON ENaC mRNA AND PROTEIN

Cloning the three channel subunits has provided specific probes for monitoring variations in channel mRNA and protein. An obvious question addressed by many investigators is the effect of aldosterone on the abundance of mRNA coding for the three channel subunits [27–40]. Surprisingly, it turned out that the three subunits are differently affected by the hormone and also that the same subunit can respond differently in different aldosterone-responsive tissues. In kidney and kidney-derived cell lines, aldosterone evokes a twofold to fourfold increase in α mRNA but does not significantly alter the abundance of β or γ [29, 31–33, 36–39]. A similar pattern is seen in lung, but in this case, the channel responds to glucocorticoids rather then mineralocorticoids [28–30]. This response is thought to play a role in lung maturation and fluid clearance after birth. In contrast to the mild effects of aldosterone on ENaC mRNA observed in kidney, in the colon, β and γENaC are strongly (≥10-fold) elevated by aldosterone, while α appears to be constitutively expressed [27, 29, 32–34, 36, 37]. The *Xenopus* kidney-derived cell line A6 shows a "kidney-like" behavior when grown on porous supports and a "colon-like" behavior when cultivated in plastic dishes [35]. It has been suggested that the large transcriptional effects of aldosterone on β and γ are needed during a high rate of replication (distal colon surface cells and A6 cells grown on plastic) but not in slowly replicating cells (kidney nephron and cells cultivated on filters) [35].

In light of the different effects of aldosterone on the three ENaC subunits, it is of interest to examine their relative importance to channel function. The current notion is that the three subunits assemble into a hetero-oligomer, which defines the amiloride-blockable pore [41–43]. Hence, it has been assumed that they are all essential and equally important components of the channel. This is also based on expression studies in *Xenopus* oocytes and mammalian cells demonstrating a very low or no channel activity in the absence of any one of the three subunits [19, 20, 44, 45]. It has also been shown that the biophysical properties of channels composed of only two subunits differ from those recorded from native epithelia or cells expressing all three components [46]. Additional support for the crucial role of each subunit is provided by studies generating ENaC knockout mice. Disrupting the gene coding for any ENaC subunit was shown to cause an early death caused by hyperkalemia and salt wasting [47–49]. αENaC knockout mice, however, die from respiratory stress, which is less severe or not detected in β and γ knockouts [47, 48, 50]. Thus, liquid clearance in lung appears to depend more on α than on β or γ.

These described effects of aldosterone on channel mRNA lend support to the notion that enhanced transcription (or inhibited degradation) of channel subunits cannot fully account for the aldosterone-induced increase in channel activity. First, it seems that induction of ENaC subunits in the kidney is far too small to account for the large increase in channel activity. Data reported in [5, 32] indicate a big quantitative difference between the effect of aldosterone (or low Na^+ intake) on the number of apical channels and the abundance of channel mRNA. That is, while the hormone evokes a dramatic (>50-fold) increase in channel density, it enhances channel mRNA by less than fourfold. Induction of β and γ mRNA observed in distal colon is, in principal, large enough to account for the hormonal effect in this tissue [29, 32, 36, 37]. However, there is a clear discrepancy between the time course of this induction and the hormonal effect on channel activity measured as amiloride-blockable Isc [23, 32]. The well-establish effect of aldosterone in distal colon and other model epithelia is characterized by a latent period of approximately 60 minutes, and the increase in channel activity can reach its half-maximal value within approximately three hours [3]. Induction of β and γENaC mRNA, on the other hand, is not apparent during the first three hours of hormonal stimulation and develops only after a longer incubation period. The finding that induction of channel mRNA lags behind the transcription-dependent increase in channel activity proves that enhanced transcription (or inhibited degradation) of ENaC subunits does not account for the early response to the hormone and may play a role only in the more chronic response. The data agree well with previous findings using the toad bladder model epithelium [14, 15, 51]. These studies have reported a similar discrepancy between the time course of aldosterone action measured in the intact epithelium and in plasma membrane vesicles or in poly A^+ RNA-injected oocytes. It fits our model that aldosterone acts on the apical Na^+ permeability by two different mechanisms: an early activation or translocation of pre-existing channels and a more chronic induction of channel protein [2, 3, 16].

It is not yet clear whether these effects of aldosterone on ENaC mRNA represent direct transcriptional regula-

tion of the channel or are secondary to the induction of other regulatory proteins. Functional analysis of putative promoter regions has been done for both α and γENaC [52–56]. For α, a 5′ flanking region that confers a glucocorticoid induction of reporter genes has been identified [53, 55]. This region includes a sequence that resembles a glucocorticoid response element, and its ability to bind glucocorticoids has been confirmed by a gel shift assay. It was also demonstrated that the Ras pathway has an antagonistic effect on the regulation of αENaC by glucocorticoids [54]. This may be a physiologically important pathway since K-Ras2A has been found to be induced by aldosterone [57, 58]. On the other hand, no region that confers transcriptional regulation by adrenal steroids could be identified in the γENaC promoter region [52, 56].

In contrast to the many studies assaying variations in ENaC mRNA in different epithelia and epithelia-derived cultures, little information is available on effects of aldosterone on the channel protein. Dijkink et al have recently reported a small aldosterone-induced increase in α and β (but not γ) protein in immunodissected rabbit kidney connecting tubule (CNT) and cortical collecting duct (CCD) cells [39]. As seen for colonic mRNA, elevation of protein synthesis appears to lag behind the increase in Isc, suggesting that it does not contribute to the initial response. On the other hand, May et al reported that in A6 cells, an increase in the rate of α synthesis is seen as early as one hour after the addition of aldosterone [35]. Since this effect precedes the increase in α mRNA, it is believed to reflect regulation of α translation by other aldosterone-induced proteins.

POSSIBLE MECHANISMS MEDIATING THE ALDOSTERONE-INDUCED INCREASE IN APICAL Na^+ PERMEABILITY

Studies summarized in the previous section confirm the notion that induction of channel subunits is not a major factor in the transcription-dependent increase in channel activity. The response to the hormone must therefore involve other induced proteins in which the function could be to modulate activity of pre-existing apical channels, alter trafficking of channel subunits between intracellular organelles and the apical surface, or even affect translation of the channel protein. Elucidating the cellular events mediating the hormonal response has been attempted in different laboratories using two complementary strategies: (*1*) the "backward approach," in which processes controlling ENaC activity were characterized, and factors involved in them were examined for aldosterone-responsiveness; and (*2*) the "forward approach," in which genes and proteins affected by the hormone were identified and studied for possible involvement in channel activation.

Several mechanisms that control the luminal amiloride-blockable Na^+ permeability of tight epithelia have been identified and characterized [reviewed in 21, 23]. These include regulation of ENaC by direct and indirect interactions with cell Na^+ and Ca^{2+}, a carboxymethylation reaction that may also involve G proteins, activation of the channel by an extracellular protease termed CAP1, and the regulation of ENaC lifetime in the apical surface through interaction with Nedd4. With the possible exception of carboxymethylation and G proteins, no data relating any of these mechanisms to the hormone-induced cascade of events have been provided thus far. In particular, neither Nedd4 nor CAP-1 appear to be affected by aldosterone [59, 60]. Some studies have suggest involvement of a methyl transfer reaction and/or G proteins in the response to aldosterone, but the exact cascade of events is far from being understood [61–64].

Much effort has been devoted to the identification and cloning of cDNA corresponding to epithelial aldosterone-induced or suppressed mRNAs. Early studies have addressed this issue at the protein level using two-dimensional gel electrophoresis of metabolically labeled proteins [65]. These and subsequent studies have identified a 65 to 70 kD aldosterone-induced glycoprotein in which the function is yet unknown. Other groups have employed various differential-screening protocols to search for aldosterone-dependent mRNAs, with special emphasis on genes induced or suppressed during the initial phase of the hormonal action. The methods employed include +/− screenings of epithelial cDNA libraries [66, 67], differential display of PCR products [57], and subtractive cDNA methods [68, 69]. These studies have resulted in the identification of a number of "early" and "late" aldosterone-dependent genes, some of which appear to provide important clues to the mechanisms of the hormonal action.

One group of induced cDNAs cloned by differential screening of rat and chicken intestine cDNA libraries code for mitochondrial oxidative phosphorylation enzymes (for example, cytochrome c oxydase and NADH dehydrogenase). Their induction by aldosterone fits well with early findings of the activation of mitochondrial enzymes and the induction of cytrate synthase [reviewed in 1]. The induction of these enzyme is needed to increase mitochondrial metabolism and to prevent limitation of active transport by the cell ATP/ADP ratio. The genes cloned so far are all coded by the mitochondrial rather than nuclear genome. This genome also appears to be a target to adrenal steroids [70–72]. Spindler and Verrey have reported that a two-hour incubation of A6 cells with aldosterone reduces the mRNA abundance of c-*myc*, c-*jun*, c-*fos*, and the glucocorticoid receptor by 50 to 80% [57, 73]. Surprisingly, the down-regulation of proto-oncogene was independent of ongoing transcription and may reflect a nongenomic effect of the steroid.

Another group of cDNAs identified as aldosterone induced code for new genes in which the functions are yet unknown. One such cDNA translates a short transmembrane protein, designated CHIF, that is specifically expressed in kidney collecting duct and distal colon [66, 74, 75]. As with β and γENaC, CHIF mRNA is strongly induced by aldosterone in the colon but is constitutively expressed in kidney [75]. Although its induction is apparent only after a prolonged incubation, CHIF can be transcriptionally elevated by corticosteroids in a time frame of 0.5 to 4 hours [76, 77]. Its cellular role is yet unknown, and some observations relate CHIF to K^+ rather than Na^+ transport [66, 78]. Two other new "early" aldosterone-induced sequences cloned from A6 cells do not appear to correspond to a typical mRNA [57].

Undoubtedly, the most promising "early" aldosterone-induced transcripts identified so far are two members of signaling pathways: the small G protein K-Ras2A and the serine/threonine kinase *sgk* [57, 68]. Most importantly, both of them appear to increase ENaC activity substantially by coexpression in *Xenopus* oocytes [58, 68, 69]. K-Ras2A is a splice variant of K-Ras2 in which the C tail contains a palmitoylation site. Its mRNA and protein are increased several-fold by the incubation of A6 cells with aldosterone for two hours, and a similar response is seen in kidneys of frogs injected in vivo with aldosterone [57, 73]. The channel activity recorded in *Xenopus* oocytes coexpressing ENaC and a constitutively active mutant of K-Ras2A is not different from that recorded from oocytes expressing ENaC alone [58]. This, however, appears to reflect compensation of the K-Ras2A–induced increase in ENaC activity by a K-Ras2A–dependent maturation of the oocytes. Normalizing the data to membrane capacitance or cell surface expression of epitope tagged ENaC demonstrates a threefold increase in specific channel activity. The identification of K-Ras2A as a channel-activating aldosterone-induced protein is in agreement with early data suggesting an aldosterone-induced guanosine 5′-triphosphate (GTP) hydrolysis [62], GTP-induced increase in channel activity [79], and a role for prenylation in the response to aldosterone [80].

The other aldosterone-induced gene likely to be involved in the regulation of ENaC codes for a member of the serine/threonine kinase superfamily. This kinase was cloned before as a serum and glucocorticoid-dependent kinase (*sgk*) in a rat mammary tumor cell line [81]. It is transcriptionally regulated by a number of other stimuli such as central nervous system injury [82], hypertonicity and secretagogues [83, 84], and heparin [85]. In A6 cells [68], rabbit collecting duct primary culture [69] and distal colon [86], *sgk* mRNA and protein are strongly induced by aldosterone. The effect is rapid, evoked by low doses of the steroid, and insensitive to translation inhibitors. Thus, *sgk* appears to be a primary aldosterone-induced gene that may be involved in the early response to the hormone. This hypothesis is strongly supported by the fact that coexpressing ENaC and *sgk* in *Xenopus* oocytes increases the channel activity by at least fourfold [68, 69, 86]. It is yet unknown whether this response is mediated by an increase in ENaC cell surface expression or a change in its open probability.

The mechanism by which *sgk* and K-Ras2A activate ENaC in *Xenopus* oocytes (and presumably also in epithelial cells) is yet unknown. One possibility is that *sgk* itself or some other downstream kinase directly phosphorylates the channel. Such a possibility is in agreement with the finding that the carboxy tails of β and γENaC undergo an aldosterone-induced phosphorylation in transfected Madin-Darby canine kidney cells [87]. Since this domain is crucial for the channel–Nedd4 interaction, its putative phosphorylation may affect the channel lifetime in the apical membrane. Obviously, many other scenarios by which a kinase signaling cascade can lead to an increase in ENaC activity are possible. Irrespective of various potential down stream events, the transcriptional regulation of *sgk* and K-Ras2A would not be sufficient to render them active, and they need to be activated by upstream effectors. For example, in other systems, *sgk* was shown to be activated (phosphorylated) by the PI-3 kinase pathway [88, 89]. This may also provide a link to the activation of ENaC by insulin [90]. Thus, much work is still needed to elucidate the cellular events relating the induction of K-Ras2A, *sgk*, and other factors to the activation of ENaC. As in other cases, the result is likely to be a complex network of signaling pathways that integrate different stimuli and affect ENaC in more than one way. Nevertheless, the previously described recent findings now provide a real chance for elucidating the mechanism by which aldosterone activates the Na^+ channel.

NOTE ADDED IN PROOF

Alvarez de lo Rosa et al (*J Biol Chem* 274:37834–37839, 1999) recently reported that *sgk* acts by increasing EnaC's cell surface expression. The response was insensitive to deletion of the carboxy termini of the channel, indicating that it is not mediated by phosphorylations within this region.

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APPENDIX

Abbreviations used in this article are: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; CAP-1, channel activating protein-1; CCD, cortical collecting duct; CNT, connecting tubule; ENaC, epithelial Na^+ channel; GTP, guanosine 5′-triphosphate; P_0, open probability; *sgk*, serum and glucocorticoid dependent kinase.

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