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Dissecting mineralocorticoid receptor structure and function☆

Fraser M. Rogerson, Francine E. Brennan, Peter J. Fuller*

Prince Henry's Institute of Medical Research, P.O. Box 5152, Clayton, Vic. 3168, Australia

Abstract

The molecular mechanisms by which aldosterone regulates epithelial sodium transport in the distal colon and the distal nephron remain to be fully elucidated. Aldosterone acts via the mineralocorticoid receptor (MR) to induce the expression of genes whose products are involved in sodium transport. The structural basis of MR interactions with aldosterone has been examined by creating chimeras of the MR and the closely related glucocorticoid receptor; we have exploited differences in ligand-binding specificity to determine the region(s) of the MR that confer aldosterone-binding specificity. These findings have been related to a three-dimensional model of the MR based on the crystal structure of the progesterone receptor. These studies have been extended to include the characterisation of interactions between the N- and C-termini of the MR. We have characterised six genes that are regulated in vivo in the distal colon and/or kidney of the rat that are directly and acutely regulated by aldosterone administration: the three subunits of the epithelial sodium channel, serum and glucocorticoid-induced kinase, channel-inducing factor and K-ras2A. These studies provide insights into the molecular pathways that mediate aldosterone-induced amiloride-sensitive epithelial sodium transport.

Keywords: Aldosterone; Cortisol; Hypertension; Spironolactone

1. Introduction

The physiological mineralocorticoid, aldosterone, acts via the mineralocorticoid receptor (MR) to regulate ion fluxes, principally in the distal nephron and distal colon [1]. The MR is a member of the steroid/thyroid/retinoid receptor superfamily of ligand-dependent transcription factors [2]. The severe salt-wasting phenotype of MR-null transgenic mice [3] demonstrates the central role of the MR in aldosterone action. In addition to the classic target tissues, kidney, colon and salivary gland, the MR is expressed in a range of tissues. These include cardiovascular tissues, where the MR appears to play a role in the pathophysiology of cardiac fibrosis [4]. In the central nervous system, MR expression is abundant in subregions of the hippocampus and hypothalamus. The MR binds not only aldosterone with high affinity, but also cortisol (corticosterone in rodents). Given the much higher circulating levels of cortisol, occupancy of the MR by aldosterone should be precluded. In the classical sodium-reabsorbing aldosterone target tissues, aldosterone specificity is maintained by the action of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which acts to convert cortisol and corticosterone to inactive 11-reduced metabolites [5]. However, in other sites such as brain and heart, where it is not protected by 11β-HSD2, the MR will act predominantly as a cortisol/corticosterone receptor.

The MR and the glucocorticoid receptor (GR) share both functional and structural homology; this relationship has been exploited in our studies of the structural determinants of aldosterone binding and activation. These structural studies have recently been extended to examine the interaction between the N- and C-terminal domains of the MR. Such an interaction has previously been shown to play a critical role in androgen receptor (AR) function. These studies are described in the first part of this paper.

Although so-called non-genomic actions of aldosterone have been described [6], an extensive range of biochemical and physiological studies suggest that the major pathway through which the MR mediates the physiological response involves the regulation of gene expression. In the second part, the present state of our knowledge of aldosterone-induced genes is discussed.

2. Mineralocorticoid receptor ligand-binding specificity

As noted above, the MR binds glucocorticoids, such as cortisol and corticosterone, with the same high affinity as it binds aldosterone. In fact, the MR has a higher affinity

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* Corresponding author. Tel.: +61-39594-4379; fax: +61-39594-6125.
E-mail address: peter.fuller@med.monash.edu.au (P.J. Fuller).

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for these steroids than does the classical GR. It is believed that the MR and GR genes arose from a common ancestor before the evolution of aldosterone [7]. It therefore appears that aldosterone evolved to bind (and activate) the MR which retained its affinity for glucocorticoids. Conversely, aldosterone has a low affinity for the GR. Antagonists such as spironolactone and eplerenone [8] have been developed, which bind to the MR and block the actions of aldosterone. Neither of these compounds binds with high affinity to the GR, although spironolactone has a high affinity for the related AR.

As its name suggests, the ligand-binding domains (LBDs) of the MR and of the GR, located at their C-termini, bind known agonist and antagonist ligands. Crystal structures of the LBD of the GR, estrogen receptor (ER) α and β, progesterone receptor (PR) and AR, bound to natural and synthetic agonists, have been solved [9]. There is a remarkable degree of conservation of tertiary structure, the backbone of which consists of 11 α-helices forming a three-layered structure with the ligand-binding pocket buried in the middle. Major insights into how steroid ligands activate steroid hormone receptors, and how antagonists block this activity, have been obtained from the crystal structures. The crystal structure of the MR LBD has yet to be solved, presumably because of the instability of the protein. The structure of the MR LBD has been modelled by us and other groups [10], based on the PR and ERα crystal structures. Although the models have provided important insights into the nature of the MR–agonist interaction, they have provided no real clues as to the determinants of MR ligand-binding specificity.

Using chimeras created between the MR and GR LBDs, we have examined the question of ligand-binding specificity of the MR. Four regions of high sequence identity were chosen as break points for the creation of the initial panel of chimeras, making a total of 16 different constructs (Fig. 1). The LBD chimeras were placed in the context of the GR N-terminal and DNA-binding domains, and were analysed in transfected CV-1 cells using a standard transactivation assay [11]. Fig. 1 shows the EC_{50} values for aldosterone activation of the chimeras. All of the chimeras activated by aldosterone contained MR sequence in the second region. Direct [^{3}H]-aldosterone-binding experiments confirmed the functional assay [11]. As expected, all of the chimeras bound [^{3}H]-dexamethasone, confirming that they are both expressed and retain the ability to bind ligand.

The second region of the initial panel of chimeras corresponds to amino acids 804–874 of the MR LBD. This region of the LBD contains two α-helices, designated H5 and H7, that form part of the ligand-binding pocket (see Fig. 2). In order to narrow the critical region within this 71 amino acid region, a second set of chimeras were created within this segment (Fig. 3). Again, this new panel of chimeras was analysed using a transactivation assay and by binding of [^{3}H]-ligands. As shown in Fig. 3, the chimera containing amino acids 820–844 of the MR LBD has the same EC_{50}

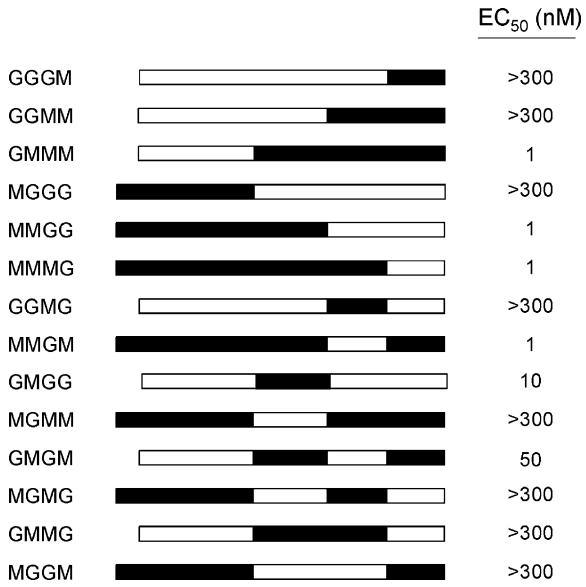

Fig. 1. Diagrammatical representation of the first series of chimeras, together with the EC_{50} values of transactivation assays using aldosterone as ligand. CV-1 monkey kidney fibroblast cells were transiently transfected with the chimera expression plasmid, an MMTV-luciferase reporter plasmid and a pRSV-β-galactosidase transfection control plasmid. The cells were incubated with aldosterone for 24 h before being harvested for assay (adapted from 11).

for aldosterone (1 nM) as the full-length MR LBD. The K_d values for [^{3}H]-aldosterone binding to the MR LBD and MR (820–844) are 0.9 and 1.3 nM, respectively, confirming the importance of this region for aldosterone binding (Rogerson et al., manuscript in preparation). This is a region of low amino acid sequence identity between the MR

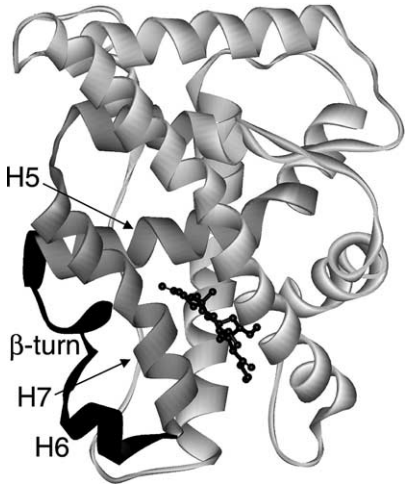

Fig. 2. Model of the mineralocorticoid receptor ligand-binding domain, based on the crystal structure of the progesterone receptor LBD [44]. The region (amino acids 804–874) found to be critical for aldosterone binding from analysis of the first set of chimeras is shown in dark grey. The region (amino acids 820–844) found to be critical for aldosterone binding from analysis of the second series of chimeras is shown in black.

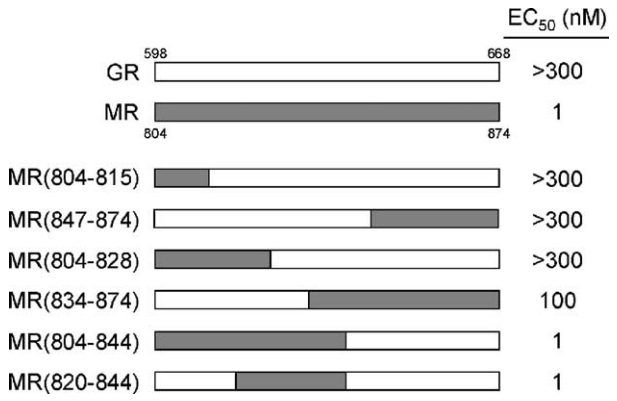

Fig. 3. Diagrammatical representation of the second series of chimeras, together with the EC_{50} values of transactivation assays using aldosterone as ligand. CV-1 monkey kidney fibroblast cells were transiently transfected with the chimera expression plasmid, an MMTV-luciferase reporter plasmid and a pRSV-β-galactosidase transfection control plasmid. The cells were incubated with aldosterone for 24 h before being harvested for assay (Rogerson et al., manuscript in preparation).

and GR. Surprisingly, this region does not form a part of the ligand-binding pocket (Fig. 2), but occurs on the surface of the receptor. How this region controls binding specificity remains to be determined and is the focus of ongoing studies.

3. Interaction between the N-terminal and ligand-binding domains of the mineralocorticoid receptor

Steroid hormone receptors are modular proteins containing four major functional domains. Although these domains have independent functions, they can also interact with one another, altering the activity of the protein as a whole. One example is the interaction between the N-terminal and LBDs (N/C interaction). This has been best described in the AR ([12] and references cited therein), although it has also been observed in ERα and the PR. In the AR, the N/C interaction contributes to homodimerisation, and its major functional consequence is to increase the stability of androgen binding to the receptor [13].

No such interaction has previously been described for the MR or GR. We used the mammalian-2-hybrid assay to examine whether an interaction occurs between the N-terminal domain and the C-terminal region (consisting of the hinge region and the LBD) of either receptor [14]. As shown in Fig. 4A, there was an aldosterone-dependent interaction of the two domains of the MR. In contrast, no N/C interaction was seen with the GR [14]. The EC_{50} of the MR N/C interaction was approximately 1–3 nM, which is similar to the EC_{50} of aldosterone-mediated transactivation in our previous studies. This result suggests that the interaction is important for MR function. Also, the interaction did not occur in the presence of the MR antagonist spironolactone which also inhibited the aldosterone-mediated interaction [14]. Intrigu-

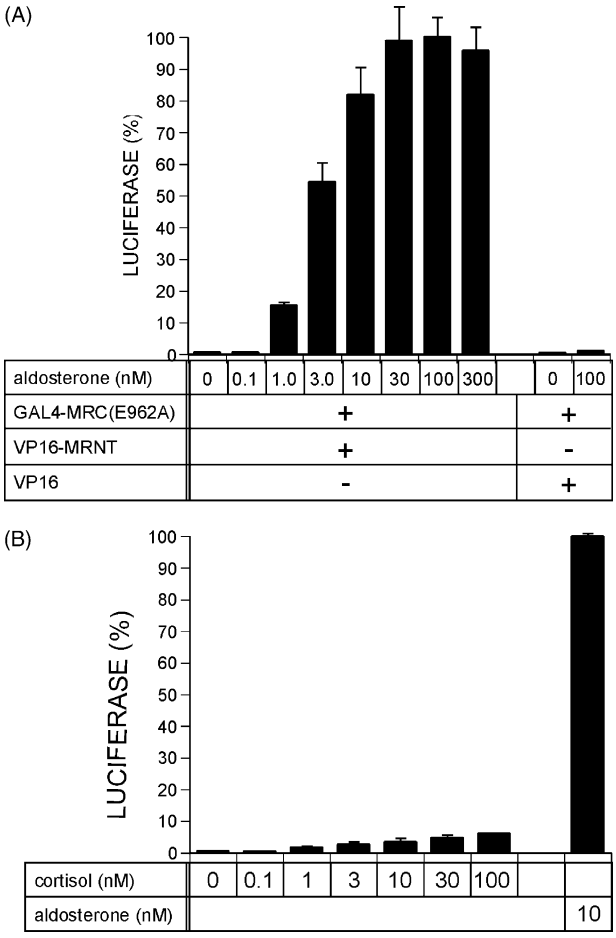

Fig. 4. Examination of interaction between the N-terminal and ligand-binding domains of the MR using the mammalian-2-hybrid assay. The N-terminal domain was attached to the activation domain of VP16 (VP16-MR NT). A mutation (E962A) in the AF-2 region of the MR LBD was created to decrease background activity in the assay, and this mutant was attached to the DNA-binding domain of GAL4 (GAL4-MRC (E962A)). COS-1 monkey kidney fibroblast cells were transiently transfected with the mammalian-2-hybrid constructs together with a GAL4-responsive luciferase reporter gene. The cells were incubated with aldosterone (A) or cortisol (B) for 24 h before assay (reproduced with permission from *Molecular and Cellular Endocrinology*).

ingly, the N/C interaction was very weak in the presence of the other major physiological ligand of the receptor, cortisol (Fig. 4B). Cortisol antagonised the aldosterone-mediated interaction of the two domains [14]. This is of interest in the light of findings in the "non-classical", non-epithelial, sites of aldosterone action; aldosterone acts in the central nervous system to raise blood pressure, the effect being antagonised by corticosterone [15]. Similarly, in rat cardiac myocytes, aldosterone, but not corticosterone, increases [^{3}H]-leucine incorporation in conditions where GR occupancy is blocked [16]. These differences may be explained, at least in part, by the lack of an N/C interaction in the presence of cortisol/corticosterone. Recently, it was found that a CREB-binding protein, CBP/RNA helicase A-containing

co-activator complex, interacts with the AF-1 region of the rat MR N-terminal domain [17]. The interaction of this complex with the MR was ligand-dependent, occurring in the presence of aldosterone but not in the presence of cortisol. These results are consistent with our findings that the role of the MR N/C interaction is significant only when the receptor binds aldosterone. In certain systems cortisol-bound MR appears to act identically to aldosterone-bound MR, whereas in other systems the effects are quite distinct. It would appear also, that the N/C interaction is involved in MR activity only in certain situations. There is precedent for this, as it has been shown that the AR N/C interaction is important for AR activation of the promoters of some androgen-regulated genes, but not others [12]. Future studies will examine the functional significance of the MR N/C interaction.

4. Aldosterone-induced epithelial sodium transport

The subcellular mechanisms by which activation of the MR results in sodium flux have, until recently, been a subject of conjecture rather than biological fact. Input from electrophysiology, biochemistry and molecular genetics has enabled identification of the key structural elements in the epithelial cell which mediate vectorial sodium transport (reviewed in [1]). Sodium entry at the luminal surface is electroneutral via the amiloride-sensitive epithelial sodium channel (ENaC). This channel consists of three closely related subunits (α, β and γ) with two transmembrane domains and an extracellular loop. Inactivating mutations of this channel in transgenic mice and in patients with pseudohypoaldosteronism results in aldosterone-resistant salt wasting [18,19]. Mutations within a proline-rich five amino acid PY motif in the intracellular C-terminus of the β- or γ-subunits result in Liddle's syndrome (pseudoaldosteronism), an amiloride- and salt-sensitive autosomal dominant form of familial hypertension in which increased channel activity is observed [19]. These observations reinforce the pivotal role ENaC plays in epithelial sodium flux. Sodium efflux from the cell across the basolateral membrane into the circulation is mediated by the ubiquitous energy-dependent sodium pump sodium–potassium adenosine triphosphatase (Na.K-ATPase).

5. Aldosterone-induced genes

The major mechanisms of sodium flux involve the MR acting through regulation of gene expression, i.e. as a transcription factor. It follows that there are aldosterone-induced genes and proteins (AIPs); these have proven elusive. Various groups have used a range of strategies and experimental systems to identify and/or characterise putative aldosterone-induced genes. Our focus has been on the early phase of the response to aldosterone in the rat distal colon in vivo. Antinatriuresis is observed in vivo with a lag of 45–90 min post-aldosterone exposure. This lag presumably reflects the time needed to elaborate the genomic response. As aldosterone exposure continues and the response matures, secondary induction of gene expression will occur. Ultimately, these responses are, for instance, reflected in the morphological changes which occur in the cells of the distal nephron after chronic aldosterone administration. Our focus is on primary response genes; i.e. those genes directly (and hence acutely) regulated by the MR. The model is a simple one: adrenalectomised male Sprague–Dawley rats are treated with a single parenteral dose of aldosterone or dexamethasone (to identify GR-mediated responses) and killed between 30 min and 4 h after the single dose. RNA is isolated from distal colon and whole kidney for Northern blot analysis or reverse transcriptase–polymerase chain reaction (RT–PCR).

6. Epithelial sodium channel

Given the pivotal role of ENaC in aldosterone-induced sodium transport, the subunit genes were strong candidates for induction by aldosterone. The α-subunit is constitutively expressed in kidney and distal colon; there is evidence that chronic aldosterone treatment increases α-subunit gene expression in some segments of the nephron, but not in the colon [1,20]. Expression of the β- and γ-ENaC subunit genes has been found to be upregulated by both acute [20] and chronic [21] aldosterone treatment in the distal colon, but not the kidney. Although the acute response occurs within 3 h, it is probably not adequate to explain the earliest phase of the aldosterone-induced increase in sodium transport [1,20].

7. Putative ENaC modifications

In view of the critical role ENaC plays in the aldosterone response, proteins that modulate channel numbers in the apical plasma membrane and/or the open probability of existing channels are potential AIP. Induction of a serine protease-like activity as a part of the mineralocorticoid response was postulated many years ago [1]. Recent studies have identified at least three membrane-bound channel-activating serine proteases [22], however, evidence for their regulation by aldosterone is lacking. Similarly, although the intracellular domains of the ENaC [23] subunits can be phosphorylated by activation of several second messenger pathways, there is no evidence that these are directly regulated by aldosterone. There is evidence from amphibian systems, principally *Xenopus* kidney-derived A6 cells, that carboxymethylation plays a role in the cellular response to aldosterone [1], though whether this involves methylation of the ENaC subunits is not clear. The serine–threonine kinase, serum- and glucorticoid-regulated kinase (sgk), is a bona fide aldosterone-induced gene as discussed below, however,

initial suggestions that it may directly phosphorylate ENaC have proven incorrect.

8. Nedd4-2

The clinical syndrome of monogenic hypertension, Liddles's syndrome, results from disruption of ENaC turnover, as described above. The turnover of the ENaC subunits is clearly an important component of their cellular regulation. Neuronal precursor cell—expressed, developmentally downregulated (Nedd4), is a ubiquitin–protein ligase originally cloned by a subtractive screen of embryonic neuronal cells [1]. WW-domains within Nedd4 bind to the PY motif in the C-termini of the ENaC subunits and promote degradation. A closely related molecule was recently identified as Nedd4-2, the original protein being designated Nedd4-1 [24]. Both contain C2/Cal B (calcium-dependent phospholipid-binding domain), three (Nedd4-1) or four (Nedd4-2) WW (protein–protein interaction domains) and HECT (ubiquitin–protein ligase domain) domains. Nedd4-1 expression is relatively ubiquitous: it overlaps with but does not parallel MR expression. Nedd4-1 gene expression is not regulated acutely by corticosteroid treatment in the distal colon [20]. Subsequent studies have identified Nedd4-2 as the isoform which regulates ENaC turnover in vivo [24]. Moreover, Nedd4-2 shows a more restricted pattern of expression than Nedd4-1 [24] with high levels in liver and kidney, moderate levels in heart and brain and low levels in lung; distal colon was not examined. We have cloned a partial rat Nedd4-2 cDNA by RT–PCR with primers designed to the mouse sequence [24] for use as a cRNA probe, as described previously for the synthesis of other cRNA probes [20,25]. Northern blot analysis reveals the expression in kidney and colon of a transcript of similar size (~3.8 kb) to that reported in the mouse [24]. RNA samples from tissues which showed corticosteroid regulation of sgk [25] were probed with the Nedd4-2 cRNA probe. No evidence of acute regulation at 30 min or one hour was observed in either tissue in response to either aldosterone (Fig. 5) or dexamethasone (data not shown) in kidney or colon. Analysis at 2 and 4 h post-treatment showed no response to the corticosteroids.

9. Sgk

In 1999, two groups identified the gene for a known serine–threonine kinase, sgk, as an aldosterone-induced gene using suppression subtractive hybridisation [26,27]. The initial studies using A6 cells and isolated cortical collecting duct cells [27] found that sgk gene expression was upregulated within 30 min by corticosteroids and that the response was superinduced by cycloheximide pretreatment. Subsequently, we demonstrated a similarly rapid response in vivo in the rat distal colon and kidney to a single dose of aldosterone or dexamethasone [25], confirmed by others [28]. In addition, we demonstrated superinduction with cyclohexide pretreatment [25]. Sgk itself requires activation by phosphorylation; this can be mediated through activation of phosphoinositide 3 (PI 3)-kinase, which has interesting implications for cross-talk between nuclear and membrane receptor-activated signalling pathways [29]. Although co-expression of sgk and ENaC in *Xenopus* oocytes resulted in clearly increased sodium flux [26,27], the nature of the interaction between sgk and ENaC has been the subject of considerable debate. Recently, two studies have reported a direct interaction of sgk with Nedd4-2 through a PY motif in sgk [30,31]. Phosphorylation of Nedd4-2 compromised its interaction with ENaC, with a consequent increase in the number of channels in the plasma membrane [30,31]. The phenotype of sgk-null mice is indistinguishable from that of their littermate controls until they are salt-restricted, when they exhibit mild aldosterone resistance [32]. This is a very different picture to that observed with the MR-null transgenic mice [3]. This observation suggests that factors additional to sgk regulate ENaC; whether this is mediated by either or both of the two related kinases, sgk2 and sgk3 or some other molecules is not clear.

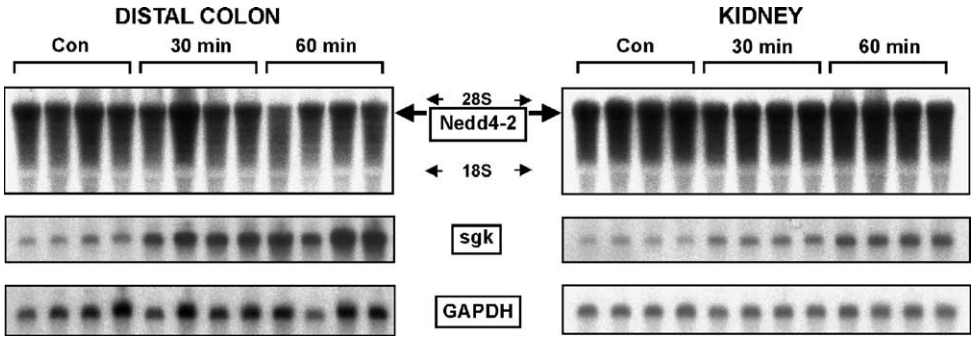

Fig. 5. Relative levels of Nedd4-2, sgk and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA in total colonic and kidney RNA 30 and 60 min after a single dose of saline, (Con) or aldosterone (50 μg/kg) administered to adrenalectomised male rats. Tissue preparation, and Northern blot analysis, together with the sgk and GAPDH cRNA probes have been described previously [25].

10. Na.K-ATPase

Although Na.K-ATPase activity in the basolateral membrane has long been known to be increased by aldosterone treatment, expression of the α- and β-subunit genes of Na.K-ATPase is not increased acutely by aldosterone [33]. The increase in activity is thought to be secondary to an increase in intracellular sodium levels, i.e. secondary to the physiological response at the apical membrane. Na.K-ATPase activity has been found to be modulated by a γ-subunit, however, levels of the γ-subunit mRNA are also not regulated by aldosterone [34].

11. Channel-inducing factor (CHIF)

Attali et al. [35] identified a novel gene, CHIF, as a putative corticosteroid-induced gene in the rat distal colon. They used a differential hybridisation approach with high dose dexamethasone treatment for 2 days. CHIF is an 87 amino acid transmembrane protein belonging to the recently identified FXYD family which includes the γ-subunit of Na.K-ATPase. CHIF is expressed in the colon and kidney, where its relative abundance, papilla ≫ medulla ≫ cortex is not as expected for an aldosterone-induced gene. Indeed, CHIF was shown to be regulated by both aldosterone and dexamethasone in the colon, but not the kidney after 3–72 h of continuous steroid exposure [36]. We have shown CHIF mRNA levels to be significantly elevated in the distal colon 60 min after a single dose of aldosterone, with a five-fold increase at 2 h; no regulation was observed in the kidney [34]. Using an intronic probe, we demonstrated that the response is transcriptional [37]; the response was also not blocked by cycloheximide pretreatment, arguing for a primary response [34]. The initial characterisation of CHIF suggested a possible role in potassium flux [35,36]. Functional analysis was not aided by the initial lack of a phenotype of the CHIF-null transgenic mouse, however, a role for CHIF in sodium transport was suggested when these mice were exposed to potassium-loading and furosemide. This physiological stress was lethal, though not to the littermate controls [38].

Beguin et al. [39] have shown that CHIF increases the Na^+ affinity of Na.K-ATPase; this is the opposite of the effect of the Na.K-ATPase γ-subunit. Thus, MR-mediated increased expression of CHIF presumably results in the early increase in Na.K-ATPase activity observed in response to aldosterone.

12. K-ras2A

Spindler and Verrey [40] found that aldosterone treatment of A6 cells for one hour increases the expression of the *Xenopus* homolog of K-ras2A. These authors also found that treatment of *Xenopus* in vivo with aldosterone increased renal K-ras2A levels at 2.5 h [40]. Stockand (reviewed in [41]) has demonstrated in a series of elegant studies that in A6 cells, K-ras2A is required for a proportion of the aldosterone-induced sodium flux. The K-ras2 gene has two transcripts that arise as a result of alternative splicing (exon 4A is either spliced in (between exons 3 and 4B) or is spliced out resulting in the K-ras2B transcript). Exons 4A and B show significant homology including a stop codon so that the resulting peptides are of the same size (21 kDa) and of identical sequence except for the C-terminal 22 amino acids. In most tissues, including rat kidney and colon (Brennan and Fuller, unpublished observations), K-ras2B is the

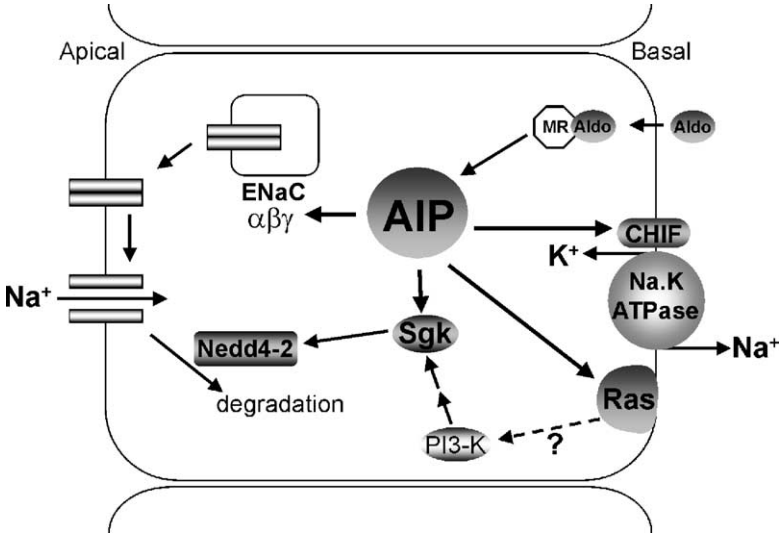

Fig. 6. Schematic representation of an aldosterone-sensitive epithelial cell indicating the postulated interaction between the putative aldosterone-induced proteins (AIP): the α, β, and γ subunits of the epithelial sodium channel (ENaC), serum- and glucocorticoid-regulated kinase (sgk), the signal transduction protein, K-ras2A and channel-inducing factor (CHIF). PI 3-K is phosphoinositide 3-kinase.

predominant isoform. As in the amphibian system, we find acute upregulation of K-ras2A mRNA levels in the rat distal colon in response to both aldosterone and dexamethasone (Brennan and Fuller, unpublished observations). K-ras2A could act through PI 3-kinase to enhance sgk phosphorylation, however, in amphibian cells the MAPK signalling pathway has also been implicated in the response to Ras upregulation [41], so other targets including perhaps ENaC itself may be influenced by K-ras2A.

13. Other aldosterone-induced genes

Recently, several other as yet incompletely characterised aldosterone-induced genes have been reported. Robert-Nicoud et al. [42] used serial analysis of gene expression (SAGE) to identify several transcripts whose mRNA levels are acutely increased by aldosterone, including glucocorticoid-induced leucine zipper (GILZ), a previously described glucocorticoid-induced gene. Boulkroun et al. [43], using a subtractive hybridisation technique, have identified *N*-myc downstream-regulated gene 2 (NDRG2) as being regulated by aldosterone, but not dexamethasone. If confirmed, this will be the first corticosteroid-regulated epithelial gene exhibiting MR specificity. The role of these two genes in the regulation of epithelial sodium transport remains to be determined.

14. Conclusions

The current state of our understanding of the molecular basis of aldosterone action is summarised schematically in Fig. 6. The molecular events involved in the binding, activation and transactivation of the MR are slowly being elucidated. The picture remains incomplete, despite the MR having been cloned 15 years ago.

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